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Hepatic transport mechanisms : the influence of bile salts

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hepatic transport mechanisms

the influence of bile salts

roel j. vonk

HEPATIC TRANSPORT MECHANISMS

The influence of bile salts

STELLINGEN

I

Bij hoge serum-galzout-spiegels kunnen bepaalde farmaka, die normaal in hoge mate door de lever worden opgenomen, een afwijkend farmakokinetisch gedrag en een afwijkend werkingspatroon vertonen.

II

De door Somogyi et al. beschreven waarneming van een verlengde werkingsduur van pancuronium in patienten met een galweg-obstruktie kan verklaard worden door de aanname dat galzouten de leveropname van pancuronium remmen.

Somogyi, A.A., C.A. Shanks and E.J. Triggs. Br. J. Anaesth. 49, 1103 (1977)

III

Het standaardizeren van termen en symbolen in de farmakokinetiek verdient aanbeveling.

IV

Wetenschappelijke prestaties in de vorm van publikaties van personen, die in de research werkzaam zijn, kunnen het beste beoordeeld worden aan de hand van de frequentie waarmee door anderen naar die publikaties verwezen wordt.

V

Door het verstrekken van stencils om kollegestof te verduidelijken of samen te vatten wordt bij de medische student een attitude gevormd waarop — in de latere huisartsenpraktijk — door folders van de farmaceutische industrie ingespeeld wordt.

VI

Het verschil in de mate van verontrusting binnen het christelijke milieu over het probleem van abortus en het probleem van oorlog en vrede is opmerkelijk.

VII

Elke eigentijdse aanpassing van de promotie-plechtigheid is in strijd met de aard van deze plechtigheid.

VIII

Uit het huidige record aantal verkopen van nieuwe auto's hoeft niet noodzakelijkerwijs gekonkludeerd te worden dat een dreigende energiecrisis is afgewend.

IX

Met betrekking tot de spreiding van rijksdiensten is het teleurstellend dat de P.T.T. haar oude slogan: "Laat eens iets van je hoorn" heeft laten vallen.

Roel J. Vonk 8 maart 1978.

Rijksuniversiteit te Groningen

HEPATIC TRANSPORT MECHANISMS

The influence of bile salts

Proefschrift

ter verkrijging van het doctoraat in de geneeskunde
aan de Rijksuniversiteit te Groningen
op gezag van de rector magnificus Dr. M. J. Janssen
in het openbaar te verdedigen op woensdag 8 maart 1978
des namiddags te 2.45 uur precies

door

ROELF JAN VONK
geboren te Baflo

VRB drukkerijen bv - Groningen

Promotor: Prof. Dr. W. Lammers
Referent: Dr. D. K. F. Meijer
Co-referent: Prof. Dr. D. D. Breimer

The investigations were carried out in the Department of Pharmacology, University of Groningen, Groningen, The Netherlands (the Supplements VII & VIII in co-operation with the Department of Zoology). The investigations have been partly supported by grants from the Netherlands' Foundation for Medical Research (Fungo).

Cover : Electronmicroscopical photograph of an isolated rat hepatocyte (dr. C.E. Hulstaert, Groningen) and photograph of a rat with a permanently implanted bile fistula
Typing : Ms. J.H. Hettema
Drawings and photos : Mr. J. Pleiter

Mijn dank gaat uit naar allen die - in welke vorm dan ook - een bijdrage hebben geleverd aan dit proefschrift.

CONTENTS

SUMMARY

The effect of bile salts on hepatic transport of drugs was investigated in male Wistar rats. Two main levels of interaction were found:

a. the biliary excretion process

b. the hepatic uptake process

The effect of bile salts on the *biliary excretion* of drugs is not uniform: the biliary excretion of some drugs is stimulated, whereas that of other drugs is not affected. Among the drugs of the latter category are compounds for which hepatic uptake is the rate limiting step in transport from plasma to bile. The mechanism of the stimulation of biliary excretion by bile salts seems to be dependent on the compound studied: the biliary excretion of the organic anion dibromosulphthalein is bile flow dependent, while biliary excretion of other organic anions like indocyanine green and rose bengal is not directly dependent on bile flow. These differences cannot be explained by qualitative differences in biliary micelle binding.

The inhibition of *hepatic uptake* of drugs by bile salts, demonstrated in intact rats, isolated perfused rat livers and isolated hepatocytes, was observed when relatively high plasma levels of bile salts (> 100 μM) were present. The hepatic uptake of both organic anions and organic cations was inhibited, which makes it less likely that only simple competition phenomena are involved. This inhibition of hepatic uptake of some drugs may have pharmacokinetical and pharmacotherapeutical consequences. This is especially so in pathological situations, when serum bile salt levels are high. This suggests that drugs, which are normally to a large extent taken up by the liver, have to be administered carefully in patients with biliary obstruction, even when these drugs are not excreted in the bile.

Under normal physiological circumstances in rats, bile salts originating from the enterohepatic circulation, stimulate the biliary excretion of some drugs, while hepatic uptake is not affected.

INTRODUCTION

The liver has many function, such as

- uptake, storage, biotransformation and excretion of endogenous and exogenous compounds (1, 2, 3)
- formation of bile
- the anabolism and catabolism of naturally occurring substrates

Hepatic function is essential for disposal of drugs and toxic agents. Compounds, taken up from the bloodstream into the liver can subsequently be metabolized, excreted in bile or transported back to the blood. Interference with one of these steps may have pharmacological consequences. In this thesis the effect of changes in a bile salt induced, bile flow on the hepatic transport of drugs is investigated.

Anatomy and physiology of the liver

The liver receives venous blood from the intestinal tract via the hepatic portal vein and a smaller volume of arterial blood via the hepatic artery. Both these blood vessels deliver their contents to the hepatic sinusoids. (Fig. 1). The organ is drained by the hepatic veins, which run into the inferior vena cava. Orally ingested substances such as nutrients, drugs and other substances must pass the liver before reaching the general circulation, because the blood circulation of the liver is situated between the intestinal tract and the general circulation

The liver is composed of various cell types, arranged in plates or laminae, that are interconnected to form a continuous three-dimensional lattice. The various cell types, schematically presented in Fig. 1 are: a. parenchymal cells or hepatocytes, which comprise about 83 % ($^{v}/_{w}$) of the liver cells (4) and are involved in most of the hepatic functions mentioned above; b. Kuppfer cells, with phagocytotic properties; c. reticulo-endothelial cells, which form the wall of the sinusoids and d. fat storing cells.

Primary bile is formed by the parenchymal cells and

excreted into the bile canaliculi, which are formed in between the parenchymal cells (Fig. 1). These bile canaliculi form a three-dimensional network and finally end up in the bile ductules.

The parenchymal cells are in open contact with the sinusoids through fenestrae in the sinusoidal walls and can take up various compounds from the blood stream. Consequently, the composition of the blood is continuously changing during passage through the sinusoids. The consequitive cells bordering the sinusoids are unequally supplied with nutrients, oxygen, endogenous and exogenous compounds, which may cause heterogeneity in the function of these cells. This heterogeneity is observed in some metabolic functions (6); evidence for hetero-

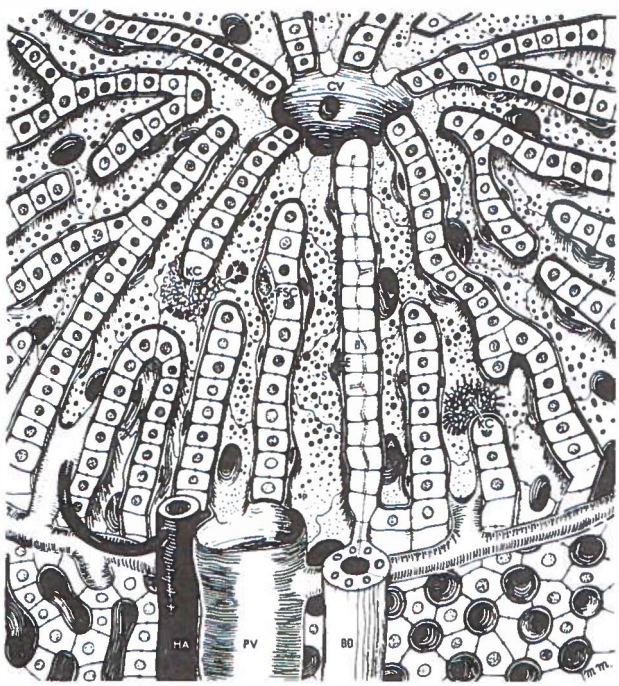

Fig. 1. Diagrammatic illustration of the three-dimensional structure of the hepatic lobule as described by Muto (5). CV central vein; KC Kupfercell; FSC fat-storing cell; B bile canaliculus; E endothelial cell; S sinusoid; L large fenestrations; SP sieve plate of small fenestrations; A anastomosing branch of a sinusoid; HA hepatic artery; PV portal vein; BD bile duct.

geneity concerning formation of bile or drug disposition is scarce or absent (7, 8).

Transport, storage and biotransformation of compounds

The liver plays a central role in pharmacokinetics, since most drugs are taken up by this organ. The rate of hepatic uptake is one of the factors determining the plasma concentration of the drug and this process may therefore be an important factor in determining the duration of action of drugs.

The mechanisms responsible for the hepatic uptake of drugs into the parenchymal cells are not well known. For the uptake of highly polar compounds, carrier proteins in the cell membranes are supposed to be required (9, 10, 11, 12, 13, 14). Once inside the hepatocyte several processes may play a role in the disposal of the drug. Drugs can be transported in an unchanged form or can be metabolized by special enzyme systems. The parent compound or the metabolites formed can be bound to intracellular binding sites, transported into the bile or transported back into the blood stream. The intracellular binding sites are located on the cytosolic proteins, the so called X, Y (ligandin) and Z proteins (15) and cell organelles (endoplasmic reticulum, mitochondria, lysosomes, nuclei). Some compounds have a high binding affinity for ligandin or arylglutathion transferase B (16) and this also applies to compounds which are not conjugated with glutathion. The cell organelles are supposed to have a high binding capacity for some drugs (17, 18). Very little is known concerning the transport processes for drugs from the hepatocyte back to plasma. It is uncertain whether carrier mediated processes or more simple diffusion processes are involved.

Drugs transported from the plasma into the bile are classified in three groups (19): a. drugs which are very poorly excreted in bile (20); b. drugs with a plasma-bile concentration gradient of about 1 (mannitol, erythritol) and which enter the bile by a passive process; c. drugs which are excreted in bile with a plasma-bile concentration ratio > 1, for instance BSP

(21), PAEB (22) and d-tubocurarine (23) and for which active transport processes are possibly involved. Smith et al. (24) concluded, that a threshold for biliary excretion of drugs exists, related to the molecular weight of that drug (about 300 for organic anions in the rat). Not all drugs share the same transport mechanisms; separate hepatic transport mechanisms for organic cations, organic anions and neutral compounds have been proposed (25). Furthermore differentiation is required for the mechanisms within the groups of organic anions (26) and organic cations (1). These particular chemical classifications should be used with caution, since the net charge of drugs depends on the biological environment (pH, ion pair binding, complex formation) and pK of the drug.

With regard to drug metabolism, the liver is involved in various biotransformations: drugs can be metabolized into pharmacologically inactive compounds, and inactive compounds can be converted into active compounds. Modification of hepatic drug metabolism can have important therapeutical consequences (27). Modifications of hepatic transport of drugs may also have pharmacological and toxicological consequences. Facilitation of the removal of (hepato)-toxic compounds from the liver into bile could have therapeutical implications. Furthermore, manipulation of the rate of biliary excretion could theoretically be used to change the duration of action of those drugs of which elimination is largely dependent on biliary excretion. Only a few cases of modification of the hepatic transport process have been reported so far. Enzyme inducing agents, like phenobarbital, stimulate biliary excretion of some non-metabolized drugs (28, 29).

Concerning the effect of changes in bile flow on biliary excretion of drugs, conflicting data have been reported. Bile salt administration to induce an enhanced bile flow, has led to contradictory results. It has stimulated (30, 31), inhibited (32, 33), or produced no effect (34, 35) on the hepatic transport of drugs. This conflict then, prompted us to investigate what the precise influence of an enhanced bile flow on biliary excretion of drugs was.

Formation of bile

After bile is secreted into the bile canaliculi, it flows into the bile duct, and where no gallbladder is present, as in the rat, the bile is excreted directly into the intestinal tract.

Part of the biliary constituents such as bile salts, phospholipids, cholesterol and proteins are present in bile in polymolecular aggregates, the biliary micelles (36). These micelles are heterogenous in size and composition and function to solubilize water-insoluble compounds in bile (37). Bile salts, having amphiphilic properties, can form micelles on their own, if their concentration exceeds a critical value (37).

At least two different anatomical sites are involved in the bile fluid formation. First, the bile canaliculi, where the primary bile is formed and second, the bile ducts, where secretion or reabsorbtion of some biliary constituents occurs. Ductular modification of bile flow can be neglected in rats (38). The canalicular bile flow can be determined by measuring the biliary clearance of mannitol (erythritol) (39, 40). Canalicular bile consists of two fractions, which are called the bile salt dependent fraction and the bile salt independent fraction. The bile salt dependent fraction is generated by a secretion of bile salts in the canaliculi, resulting in water transport due to the osmotic activity of bile salts. Drugs, excreted in bile, also have a potency to stimulate bile production by their osmotic activity. The origin of the so-called bile salt independent

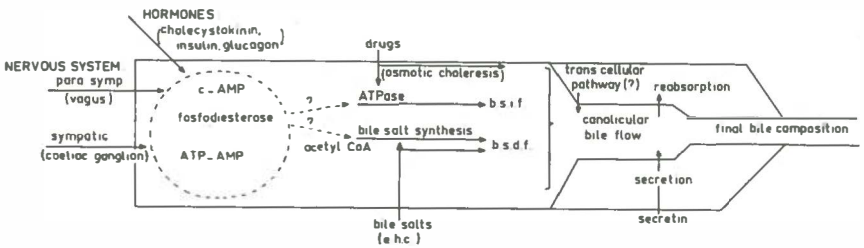

Fig. 2. Schematic presentation of factors, which may influence bile fluid formation; b.s.i.f. bile salt independent fraction; b.s.d.f. bile salt dependent fraction; e.h.c. enterohepatic circulation.

fraction is assumed to be related to sodium transport mediated by the canalicular membrane bound sodium-potassium ATPase. However, objections against this theory exist (41, 42). Other factors may possibly affect bile production, such as cholinergic and adrenergic nervous influences, cyclic AMP, but the results are as yet uncertain. A scheme of factors (possibly) influencing bile production is presented in Fig. 2. The data are derived from recent reviews concerning the physiology and pharmacology of bile fluid formation (43-46).

As indicated above an enhanced bile flow in rats can be induced by stimulation of the bile salt dependent fraction, stimulation of the bile salt independent fraction or an osmotic choleresis. Stimulation of the bile salt dependent fraction can be induced by exogenous bile salt administration or manipulation of the bile salt synthesis, both of which are used in these studies.

After bile salts are transported into the intestinal tract, they are reabsorbed from the small intestine into the blood and carried to the liver via the hepatic portal circulation. The bile salts are normally cleared from the blood with a high efficiency and again excreted into bile, resulting in an enterohepatic circulation. It is not known if the normally circulating bile salts have any effect on hepatic functions such as metabolism and transport of drugs. It is possible, that effects of bile salts on hepatic transport could well be expected especially in pathological situations, for instance hepatites or cholestasis, when high serum bile salt levels occur (47). Therefore, the second aim of this study is an investigation of the influence of bile salts on hepatic transport of drugs. Variations in bile salt supply were caused by exogenous administration of bile salts, interruption of the enterohepatic circulation or variations in hepatic bile salt synthesis.

The anabolic and catabolic function

The biochemical synthesis and breakdown of many compounds is regulated in the liver (glycogeen, fatty acids, amino acids).

The synthesis of bile salts involves the formation of cholesterol from acetyl CoA with the rate limiting enzyme hydroxymethylglutaryl CoA reductase. Cholesterol is subsequently converted into the bile salts cholate and chenodeoxycholate via the common intermediate 7 α-hydroxy-cholest-4-en-3-one (Fig. 3) (48, 49). Other, secondary bile salts, such as deoxycholate and lithocholate, are formed by intestinal microorganisms.

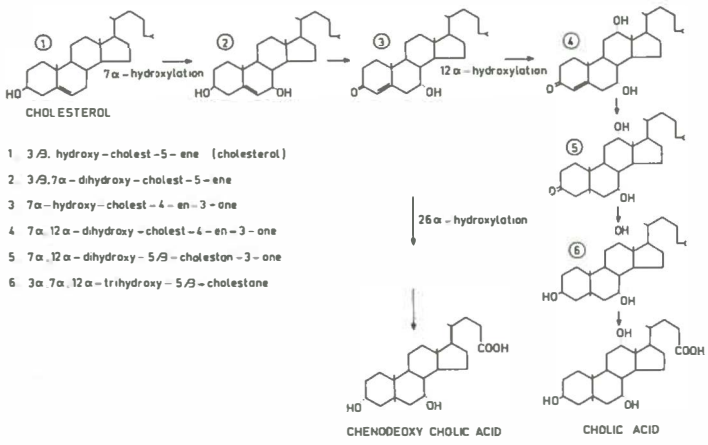

Fig. 3. Biosynthesis of the bile acids chenodeoxycholate and cholate with cholesterol as precursor.

The following chapter discusses and summarizes the results of the investigations concerning the two points raised above, that is

- the influence of an enhanced bile flow on biliary excretion of drugs,
- the influence of bile salts on hepatic transport

The experiments investigating these two points are described in the Supplements I-VIII.

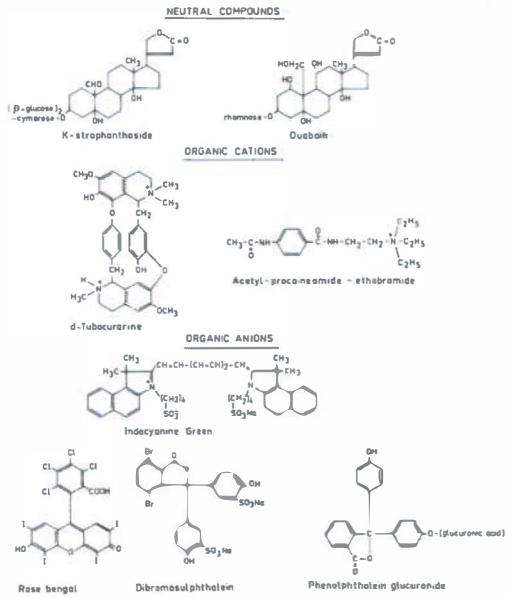

Fig. 4. Structural formulae of the drugs used.

INFLUENCE OF BILE SALTS ON BILIARY EXCRETION AND BILIARY MICELLE BINDING OF SOME DRUGS

drug	amount (μmoles/kg)	INFLUENCE CHOLERETICS		relative biliary micelle binding (%)
		taurocholate 106 μmoles/h	dehydrocholate 106 μmoles/h	
ouabain	0.29	0	0	29
K-strophanthoside	0.29	0	0	29
APAEB	35.3	0	0	25
dTc	4.2	0	0	59
ICG	12.9	++	+	
DBSP	150	+	++	65
PG	200	0	0	
Rose Bengal	12.9	+	+	

+ = stimulation
0 = unchanged

Table I. Effect of bile salt administration (106 μmoles/h of taurocholate or dehydrocholate) on biliary excretion of various drugs and the biliary micelle binding of some of those drugs.

RESULTS AND DISCUSSION

1. *Effect of bile salts on the biliary excretion of drugs*

The first series of experiments were concerned with the influence of an enhanced bile production on biliary excretion of various drugs in intact rats. The bile salts taurocholate and dehydrocholate were used to enhance bile flow. The mean increase in bile flow during administration of 106 μmoles/h of taurocholate or dehydrocholate was 60 % and 137 % respectively. Bile, obtained after afministration of both bile salts, was analyzed (Supplement VI). The total bile salt output during taurocholate and dehydrocholate administration was 101 and 108 μmoles/h respectively, whereas in control experiments 10 μmoles/h of bile salts was excreted. Taurocholate was excreted in bile in an unchanged form, while dehydrocholate was metabolized prior to biliary excretion. These metabolites were identified as monohydroxy, dihydroxy and trihydroxy steroid compounds by combined gaschromatography-mass spectrometry (Supplement VI).

The biliary excretion of the following compounds was studied two neutral compounds - the cardiac glycosides ouabain and K-strophanthoside; two organic cations - d-tubocurarine and acetylated procainamide ethobromide; and four organic anions - indocyanine green, β-D-phenolphthalein glucuronide, rose bengal and dibromosulphthalein (structural formulae Fig. 4). These compounds were chosen as they are not noticeably metabolized in rats. This considerably facilitates the interpretation of these studies.

The influence of taurocholate or dehydrocholate induced choleresis, on the biliary excretion of the above-cited compounds is presented in Table I and described in detail in the Supplements I-IV. No uniform effect of choleresis was observed whereas the biliary excretion of ouabain, K-strophanthoside, d-tubocurarine, acetylated procaineamide (APAEB) and β-D-phenolphthalein glucuronide was not stimulated by bile salt administration, that of indocyanine green (ICG), rose bengal and dibromosulphthalein (DBSP) was markedly increased. To elucidate the diffe-

rences between the choleresis effect on the biliary excretion of these drugs, first the stimulatory effect of bile salts was analyzed.

The following hypotheses have been advanced to explain the stimulatory effect of bile salt induced choleresis on the net transport of drugs from liver to bile:

1. the biliary excretion of a particular drug is bile flow dependent (30) (*choleretic effect*)
2. the biliary excretion of a particular drug is regulated by specific bile salt effects. These effects are supposed to be either (2a) direct effects of bile salts on the canalicular transport process (50). (*canalicular membrane effect*) or, (2b) are suggested to be due to biliary micelle formation by bile salts, leading to increased binding of the particular drugs in bile (*micellar sequestration*) (51, 52). In contrast to hypothesis 1, the stimulatory effect is not necessarily related to bile flow in hypothesis 2.

The experimental techniques, used to test the above mentioned hypotheses were:

a. determination of the extent of micellar binding of drugs in relation to the effect of bile salts on their biliary excretion (Supplements I, II, III, IV, VI)
b. manipulation of bile flow by various bile salts, both in intact rats and in isolated, perfused rat liver experiments (VI)
c. manipulation of bile flow by using non-bile salt choleretics (VI) again both in intact rats and in isolated perfused rat liver experiments
d. isolated hepatocyte suspensions, used to study possible membrane effects of bile salts on the transport systems without having any bile flow effect of bile salts (V)
e. variations in bile flow, resulting from the circadian rhythm and food deprivation in freely moving unanesthetized rats (VII, VIII).

a. Micellar binding of drugs in relation to their biliary excretion was investigated. In Supplement I, it is shown that

the stimulation of biliary ICG excretion by taurocholate is more pronounced than that caused by dehydrocholate, despite the higher choleresis induced by the latter bile salt. The same phenomenon was observed with rose bengal (unpublished results; 53). Taurodeoxycholate, which only very slightly stimulated bile flow, increased biliary excretion of rose bengal more than dehydrocholate did.

Because administration of taurocholate and taurodeoxycholate also stimulate biliary micelle formation, whereas dehydrocholate, for instance, is much less potent in doing so (VI), micelle binding of drugs was also studied. This was performed by ultracentrifugation of the bile, containing the drugs under study. In Supplement II the binding of three organic anions: dibromosulphthalein, ICG and β-D-phenolphthaleinglucuronide was studied. In Supplements III, IV and VI more quantitative studies investigating the binding of the two cardiac glycosides ouabain and K-strophanthoside, the organic cations APAEB and d-tubocurarine and the organic anion DBSP, were made. The relative binding of these drugs to biliary micelles, after 17 h of centrifugation, is presented in Table I. It can be concluded, that no obvious correlation between the extent of biliary micelle binding and stimulation of biliary output is present.

b. The effect of a choleresis, induced by various bile salts, on biliary excretion of dibromosulphthalein (DBSP), is described in Supplement VI. The influence of seven different bile salts was investigated in vivo. A high correlation ($r = 0.97$) between bile flow and the maximal biliary transport maximum of DBSP was found in these experiments. Taurodeoxycholate, which only very slightly stimulated bile flow, did not influence the biliary excretion of DBSP. Biliary output of DBSP under the influence of taurocholate and dehydrocholate was not related to the bile salt output per se.

c. The effect of a choleresis, induced by non bile salt choleretics, on biliary excretion of DBSP is described in Supplement VI. The administration of ouabain, which is excreted in bile in an

unchanged form (54), caused an increase in biliary DBSP excretion. The administration of ethacrynic acid and theophylline, both of which are excreted in bile as organic anions (55, 56) did not stimulate DBSO output. It was also found that DBSP and ^{14}C-ethacrynic acid inhibit each other during their biliary excretion, whereas DBSP does not influence taurocholate excretion. Thus, when choleresis is induced by anionic, non-bile salt choleretics, the possible stimulatory effects of an enhanced bile flow may be masked by competitive inhibition.

d. Isolated hepatocyte suspensions were used, to study the influence of bile salts on uptake and release of some compounds and the results are described in Supplement V. In isolated hepatocytes, possible canalicular membrane effects of bile salts should still be present, whereas choleretic effects of bile salts are absent. The isolated hepatocytes were shown to be capable of taking up and releasing compounds. The release process showed transport characteristics comparable with those of the biliary excretion process in vivo experiments (V).

Taurocholate, which was added to the incubation medium in a concentration of 1.0 mM, did not affect the release of DBSP, ICG and acetylated procainamide ethobromide from the cells. This result does not support the contention that bile salt effects on the canalicular membrane play a major role in the stimulatory effect on the biliary excretion of dyes.

e. The variations in bile flow, resulting from the influence of food deprivation and circadian variation, were also used to study the biliary excretion of DBSP. The results are given in Supplements VII and VIII. Biliary excretion of DBSP was measured at two different times of the day - night cycle; at 0.00 h when the bile flow was at its maximum and at 12.00 h, when bile flow was at its minimum. DBSP excretion was also studied after a 48 h of fasting at 12.00 h, when bile flow was minimal. It was found that DBSP excretion was highest in the periods of maximal bile flow, which confirmed the hypothesis that biliary DBSP excretion is dependent on bile flow and/or bile salt output.

The results described above lead to the conclusion that the nature of the stimulatory effect of bile salts depends on the dye used: biliary DBSP excretion is a bile flow dependent process whereas the biliary excretion of indocyanine green and rose bengal is not. Furthermore, biliary micelle binding was not found to be a pertinent factor in the biliary excretion process. This is in agreement with the recent studies of Delage et al. (57).

At present no definite explanation for the differences in effect of bile salts on biliary excretion of DBSP and indocyanine green or rose bengal can be given. However, the following speculations may be made:

- not all compounds are transported by the same type of hepatocytes (heterogeneity in transport function of hepatocytes, see page 10). If this is the case then the pattern of interaction of bile salts and drugs is also influenced by their respective distribution within the liver lobule.
- in contrast to DBSP, large amounts of compounds such as ICG, rose bengal may precipitate in the biliary compartments exogenously administered bile salts prevent the precipitation of such compounds. Such a mechanism was proposed for the reduction of the cholestatic effects of lithocholate by concomittant infusion of taurocholate (58, 59).

The correlation between bile flow and biliary excretion of DBSP, observed in our studies during bile salt induced choleresis ouabain induced choleresis and circadian variations in bile flow and also the lack of effect of bile salts on the release of DBSP in isolated hepatocytes are in line with the hypothesis of a bile flow dependent mechanism put forward by others (30, 31, 60, 61, 62). The discrepancy in the data, which invalidates the concept of a bile flow dependent mechanism (51, 52, 63, 64, 65) is discussed in Supplement VI.

One of the mechanisms, underlying the bile flow dependent, biliary excretion process, extensively discussed in Supplement VI, may be a reduced reversed transport of drugs from bile by choleresis. Consequently, if this reversed transport from bile

is absent, no effect of choleresis on biliary excretion of drugs can be expected. This may in fact occur with ouabain, K-strophanthoside, β-D-phenolphthalein glucuronide and a number of organic cations: d-tubocurarine, procainamide ethobromide (PAEB), and deptropine methobromide. As shown in Table I and Supplements II, III, IV the biliary excretion of all these compounds is not stimulated by choleresis. For APAEB another explanation for the lack of effect of the choleresis was found. Pharmacokinetic studies of equimolar doses of PAEB and APAEB suggest that the hepatic uptake of APAEB is the rate limiting step in the hepatic transport process. This could also explain the absence of effect of choleresis on its biliary excretion. Further studies have to be performed, however, before a definite model can be formulated for the interactions of drugs and bile salts in the biliary excretion process.

2. *Effect of bile salts on the hepatic uptake of drugs*

It was found (V), that addition of taurocholate (1.0 mM) to an incubation medium containing isolated hepatocytes, inhibits cellular uptake of some drugs which have largely different chemical structures. This inhibition occurred with two organic anions (ICG and DBSP) and an organic cation (APAEB). If we assume, that organic cations and organic anions are not transported by the same carrier (25), this suggests that this effect is not due to simple competition phenomena. The nature of the inhibitory effect is discussed in Supplement V. The inhibition of hepatic uptake of APAEB is not complete, in contrast to the uptake of DBSP. This suggests two different driving forces for the hepatic uptake of APAEB, one of which can be inhibited by bile salts. Schanker (66) also proposed two simultaneously operating mechanisms for the hepatic uptake of APAEB.

The inhibitory effects of bile salts was also observed in vivo (IV) and in isolated, perfused rat liver experiments (IV, VI). In isolated, perfused rat livers high doses of bile salts retarded the disappearance of DBSP from the medium. Simultaneously the slope of the biliary excretion curve remained similar,

indicating that the biliary excretion process was not affected (VI). The concentration of taurocholate which causes inhibition in isolated, perfused rat liver experiments, was in the same order as observed in experiments with isolated hepatocytes (V).

Inhibitory effects of bile salts on the pharmacokinetics of the organic cation d-tubocurarine were also observed (IV). High doses of dehydrocholate inhibited hepatic uptake of d-tubocurarine. This caused a retarded plasma disappearance, higher plasma levels and an increased renal excretion of d-tubocurarine (IV). Such increased plasma levels of d-tubocurarine may also cause a prolonged duration of action of the neuromuscular blocking agent. Recently it was reported by Somogyi (67) that, in patients with biliary obstruction, the duration of action of pancuronium was prolonged. Westra et al. in our laboratory observed the same phenomena with pancuronium and gallamine in the cat (unpublished observations). Thus, bile salts may change the pharmacokinetics of some drugs which are normally taken up by the liver. These studies suggest that in pathological situations with high bile salt serum levels, for instance, cholestasis (47) and biliary obstruction, a deviating pharmacokinetic and pharmacotherapeutic behaviour of some (polar?) drugs can be expected. This may apply to those drugs, which are normally to a large extent taken up by the liver, such as drugs undergoing "first pass" hepatic clearance, including drugs which are poorly excreted into bile. If our data can be extrapolated to the situation in man, administration of these drugs to the above mentioned types of patients has to be carefully monitored. A study on pharmacokinetics of DBSP in patients with cholestasis is currently in progress (J.H.P. Wilson, Erasmus University, Rotterdam).

The retarded plasma disappearance of BSP, DBSP or radioactive labelled bile salts, used for liver function tests in hepatic failure (68, 69, 70, 71) may be caused by a deficient transport system. It may also be the result of high bile salt levels, which inhibit the hepatic transport systems. It is unknown whether high bile salt levels inhibit hepatic uptake of endogenous compounds (amino acids, sugars etc.).

3. *Effects of bile salts on intracellular binding sites for drugs*

The third level of interaction between drugs and bile salts, may be the intracellular binding sites. Drugs taken up by the liver are bound to cytosol proteins and presumably to cell organelles. In vitro, after homogenization in a buffer, considerable amounts of drugs were found to be bound to cellular particles (IV & VI). Theoretically, drugs can be displaced from their intracellular binding sites by bile salts. Contradictory results were reported: binding of BSP to cytosol proteins in vitro was affected by bile salt administration (72), but binding of BSP (15) and ICG (73) to ligandin was not altered by bile salts. DBSP was only slightly displaced from its binding to ligandin and Z protein after adding ten times as much taurocholate (VI). It was reported (74) that bile salts reduce the so called storage capacity of bromosulphthalein. This is not necessarily a displacement of the bromosulphthalein from the intracellular binding sites, but may also be caused by a reduced concentration ratio unbound drug in plasma/unbound drug in cytosol. In conclusion, displacement of drugs by bile salts from binding sites at cell organelles is a possibility and deserves more detailed investigation in the future.

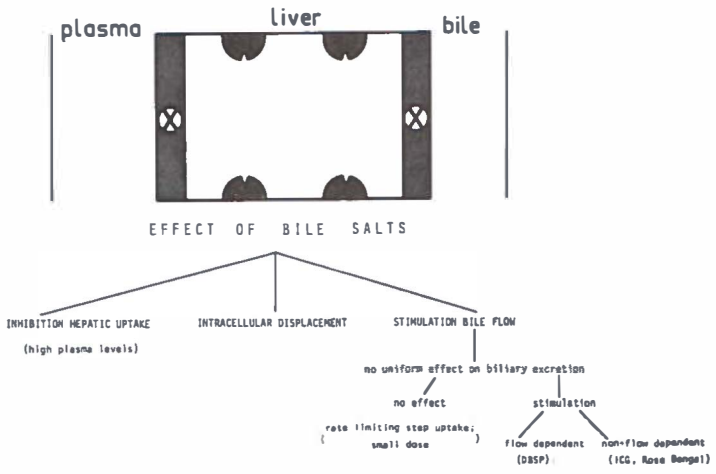

Fig. 5. Schematic presentation of the various effects of bile salts on hepatic transport of drugs.

4. *Effect of bile salts originating from the enterohepatic circulation*

As mentioned before, bile salts in relatively low concentrations may have stimulatory effects on the biliary excretion of drugs; higher concentrations have inhibitory effects on hepatic uptake of drugs, whereas intracellular displacement by bile salts in the case of DBSP is probably of less importance. These possible interaction sites are schematically represented in Fig. 5. The effect of endogenous bile salts, originating from the enterohepatic circulation, on hepatic transport of drugs was investigated by studying the pharmacokinetics of DBSP in three groups of rats: animals with a permanent enterohepatic circulation of bile salts (*permanent bile fistula rats*); animals where the enterohepatic circulation of bile salts was interrupted for about one and a half hours (*acute bile fistula rats*); and animals with a complete enterohepatic circulation of bile salts (*non-bile fistula rats*) (VIII). In the first two groups, both plasma disappearance and biliary excretion were measured, whereas in the third group only plasma disappearance could be determined. The primary hepatic clearance constant was not significantly different in the three groups of rats. This indicates that bile salts neither stimulate hepatic uptake of DBSP as suggested by others (75), nor inhibit hepatic uptake of DBSP. The normal bile salt level of the hepatic portal blood in rats is about 60 μM (76). According to our studies in isolated hepatocytes (V), this concentration would be expected to cause only a small inhibitory effect on the hepatic uptake of DBSP. The studies reported in Supplement VIII indicate that the transport maximum of biliary excretion of DBSP was closely related to bile flow and/or bile salt output. This is consistent with the proposed, bile flow dependent hypothesis. Interruption of the enterohepatic circulation of bile salt results in a lower bile flow and decreased biliary excretion of DBSP. This suggests that, under physiological circumstances, bile salts have a function in facilitating the biliary transport of some drugs.

Some other topics, not directly related with the influence of bile salts on hepatic transport of drugs, are discussed in the Supplements:

- micellar binding of endogenous biliary components (III, VI)
- micellar binding of mannitol (III)
- polarity of isolated hepatocytes (V)
- effect of collagenase treatment on isolated hepatocytes (V)
- circadian rhythm of bile flow, bile salt output and cholate/chenodeoxycholate ratio in bile (VII)
- relation between food intake and bile flow (VII)
- influence pentobarbital anesthesia on hepatic transport of DBSP (VIII)

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SUPPLEMENT I

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The Influence of Taurocholate and Dehydrocholate Choleresis on Plasma Disappearance and Biliary Excretion of Indocyanine Green in the Rat

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Summary. The pharmacokinetics of indocyanine green (ICG; 3.9 μmoles/kg and 12.9 μmoles/kg) were investigated in rats given infusion of either saline, taurocholate (106 μmoles/h) or dehydrocholate (106 or 268 μmoles/h). During the infusion of saline and taurocholate the plasma concentration of ICG decreased in a mono-exponential manner. However, with dehydrocholate the clearance of ICG from plasma showed two phases with different half lives. The half life of the rapid component (2.2 min) was about the same as the one found in the control experiments.

After injection of 12.9 μmoles/kg ICG the biliary excretion of the dye increased by 138% during taurocholate administration, while an equimolar dehydrocholate infusion resulted in a mean increament of 55%. Under these circumstances the bile flow was stimulated by 195% and 297% resp.

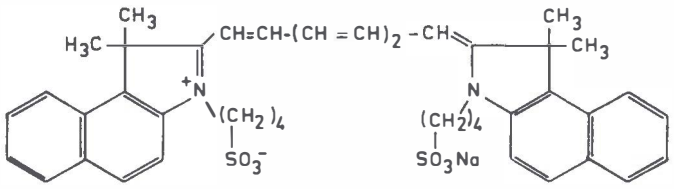
With the lower dose of ICG (3.9 μmoles/kg) however, there was no stimulation of the biliary ICG excretion with taurocholate. At this dose level an infusion of dehydrocholate (106 μmol/h) enchanced the biliary output of ICG by approximately 54%, while administration of 268 μmol/h resulted in a slight but significant decrease of 31%.

These observations can be explained by assuming interaction of the bile acids with the hepatic transport of ICG at different sites. The appearance of the second component of the plasma curve during dehydrocholate infusion is possibly related to a diminished hepatic storage capacity for ICG and is not due to an effect on the primary hepatic uptake or biliary output of the dye.

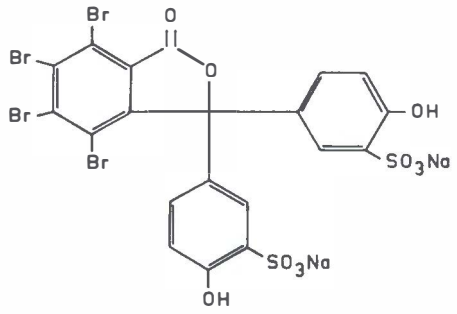
Key words: Biliary Excretion — Choleresis — ICG — Hepatic Uptake Mechanisms — Bile Acids — Dehydrocholic Acid.

Indocyanine green (ICG, Fig. 1) is considered an excellent agent for studying hepatic function. (Fox *et al.*, 1957; Wheeler *et al.*, 1958; Ketterer, 1960). It is taken up exclusively by the liver and does not undergo enterohepatic circulation nor extra hepatic elimination. The dye is not metabolized and is excreted extensively in bile. (Wheeler *et al.*, 1958; Cherrick *et al.*, 1960; Hunton *et al.*, 1960). For these reasons it gained wide popularity for diagnosis of liver diseases, along with sulphobromophthalein (BSP, Fig. 1).

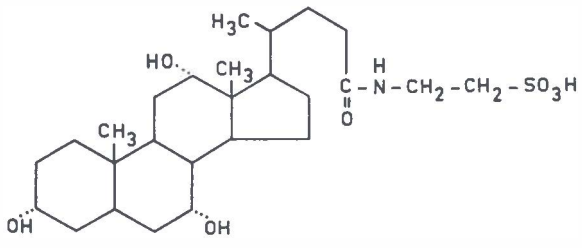
It was demonstrated in earlier studies that BSP excretion in bile could be enhanced by infusion of certain bile acids (O'Maille *et al.*, 1966;



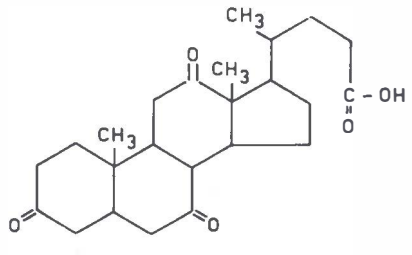
ICG indocyanine green m.w. 775



BSP sulfobromophtalein sodium m.w. 838



TC taurocholic acid m.w. 516



DHC dehydrocholic acid m.w 403

Fig. 1. Structural formulae

Ritt and Combes, 1967; Boyer *et al.*, 1970; Erlinger and Dumont, 1973; Forker, 1973). Winkler *et al.* (1970) studied the excretion of ICG in pigs during dehydrocholate and cholic acid choleresis and found only a slight stimulation of the biliary excretion of ICG by cholic acid. No further information is available concerning the influence of bile salt choleresis on the hepatic transport of the dye. Therefore it seemed of interest to include ICG in our investigations of the hepatic transport of various drugs. (Meijer *et al.*, 1970, 1971, 1972).

To induce choleresis, we used taurocholate, a natural bile constituent, which forms micelles in the bile and dehydrocholate, a semi-synthetic bile acid, which is metabolized in the liver. (Hardison, 1971; Gérolami, 1972). Only one of its metabolites (5% of the total number of moles) is able to form micelles. The present investigations was concerned with the influence of these choleretics on the pharmacokinetics of ICG.

Materials and Methods

a) Chemicals. ICG was obtained from Hynson, Westcott and Dunning, Inc., Baltimore U.S.A.

Sodium dehydrocholate and sodium taurocholate were purchased from Fluka, A.G., Buchs, Switzerland.

Solutions for infusion were prepared in saline (0.9% w/v NaCl).

b) Chemical Analysis. ICG in plasma and bile was estimated spectrophotometrically.

Plasma: blood samples were collected in heparinized tubes. After centrifugation (1700 g, 15 min) 100 µl of plasma were diluted with distilled water and the E 800 was measured spectrophotometrically. The lowest concentration of ICG which could be detected reliably using this method was 3,9 µmoles/ml, its absorbance being more than three times the value of blank plasma.

Bile: 25 µl was added to 25 µl 4% bovine serum albumin and diluted with distilled water; the E 800 was measured.

Neither dehydrocholate nor taurocholate interfered with the determination of ICG in plasma and bile.

c) Experiments in Rats. Male Wistar rats (280—320 g), which had free access to food and water, were anesthetized with pentobarbital sodium 60 mg/kg i.p. and artificially respirated during the experiment. The temperature was maintained at 37.5—38.0°C with a heating lamp. The bile duct was cannulated with polyethylene tubing. The renal vessels were ligated to facilitate comparison with earlier BSP experiments (unpublished results). Bile was collected for 10 successive 10 min periods after starting the infusion. Blood pressure was monitored and blood samples were taken from the carotid artery. Infusions (3.8 ml/h) were given via the jugular vein by means of a Braun (Melsungen) constant infusion pump. Infusions were started when normal body temperature and blood pressure were established. Constant infusions of bile salts were preceded by a priming dose of 0.5 ml of the infusion solution; in this way, 40 min after starting the infusion, bile production was fairly constant. Neither a long-term change of blood pressure nor an enhanced hemolysis due to the infusion of bile acids could be detected. At $t = 40$ min a single injection of ICG was given via the penis vein. Up to six blood samples, each of 0.3 ml, were taken and fluid loss was compensated by injection of a similar volume of saline.

Results

a) Plasma Concentrations of ICG. Disappearance of ICG from the plasma after a single injection of 12.9 μmoles/kg during infusion of saline, taurocholate (106 μmoles/h) and dehydrocholate (106 μmoles/h; 268 μmoles/h) is shown in Fig. 2. The plasma concentration is plotted on a semi-logarithmic scale. In control experiments a straight line was obtained, indicating a mono-exponential elimination with a $t_{1/2}$ of 2.2 min

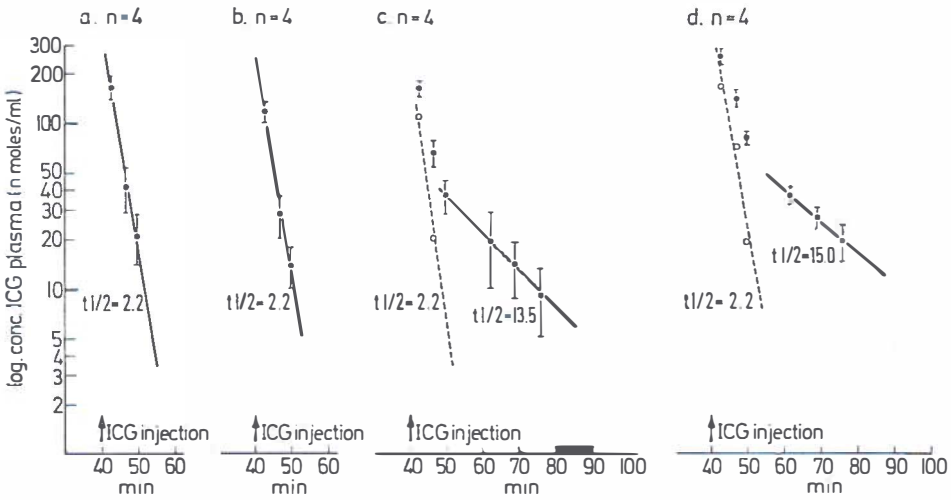


Fig. 2 a—d. Plasma disappearance of ICG during an infusion of a. saline (3.8 ml/h), b. taurocholate (106 μmoles/h), c. dehydrocholate (106 μmoles/h) and d. dehydrocholate (268 μmoles/h) after i.v. injection of ICG (12.9 μmoles/kg). Mean value of 4 experiments ± s.e.m.

Table 1. ICG excretion (nmoles/min/kg b.w.) and bile production (mg/min/kg b.w.) during infusion of saline, taurocholate (106 μmoles/h) and dehydrocholate (106; 268 μmoles/h) after ICG injection (3.9; 12.9 μmoles/kg). Mean value of six periods of 10 min after ICG injection. The ICG excretion and bile production during bile salt infusions are compared with the ICG excretion and bile production during saline infusion; in each case the stimulation c.q. inhibition is given

Infusion	ICG 3.9 μmoles/kg		ICG 12.9 μmoles/kg	
	ICG excretion	Bile production	ICG excretion	Bile production
Saline	32	63	50	40
Taurocholate	30	80	118	118
106 μmoles/h	− 6%	+ 27%	+ 138%	+ 195%
Dehydrocholate	50	140	78	159
106 μmoles/h	+ 56%	+ 122%	+ 55%	+ 297%
Dehydrocholate	22	236	72	200
268 μmoles/h	− 31%	+ 274%	+ 45%	+ 400%

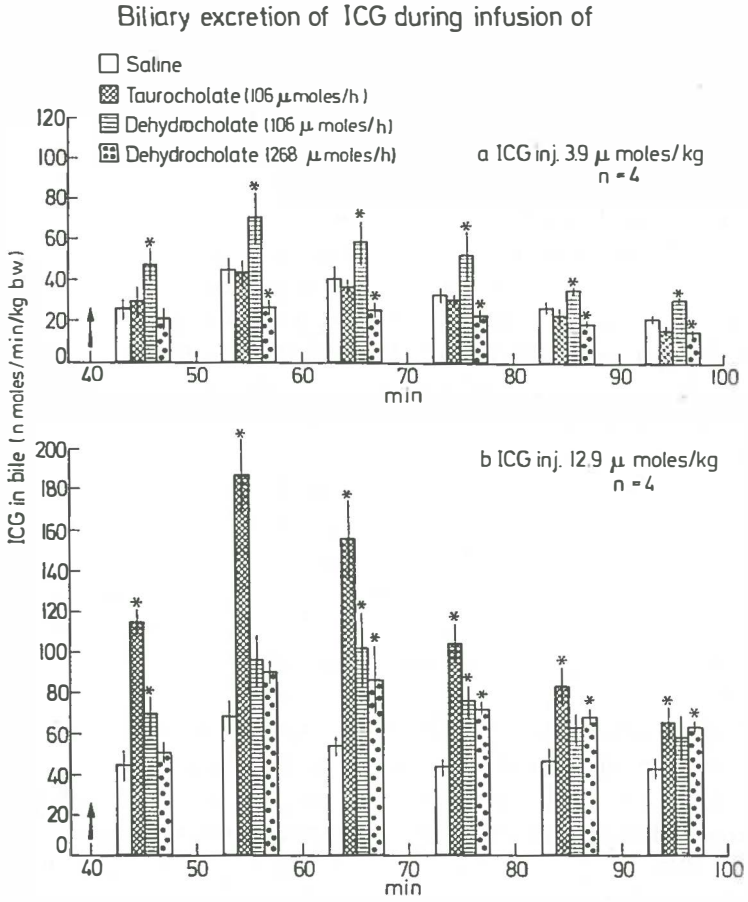


Fig. 3 a and b. Biliary excretion of ICG during six periods after ICG injection of a, 3.9 μmoles/kg and b, 12.9 μmoles/kg during infusion of saline (3.8 ml/h), taurocholate (106 μmoles/h) and dehydrocholate (106; 268 μmoles/h). As indicated in the figure ICG was injected 40 min after starting the infusion (see Methods). Mean value of 4 experiments ± s.e.m. *: significant deviating from controls. ($P < 0.05$, student t-test)

(Fig. 2a). Fifteen min after injection the concentration had fallen to a very low level. A similar pattern was reported earlier by Klaassen (1969). During taurocholate infusion a very similar plasma curve was found with the same $t_{1/2}$ (2.2 min).

However during the infusion at low dehydrocholate concentration the plasma disappearance of ICG was not mono-exponential; a second phase was present with a $t_{1/2}$ of 13.5 min (Fig. 2c). This second component was even more pronounced in the experiment using a dehydrocholate infusion of higher concentration (Fig. 2d) ($t_{1/2} = 15.0$ min). During this experiment the second phase was initiated at a higher plasma concen-

tration. The half life of the first phase (2.2 min), found after correction for the second component, was similar to the half life found in the control experiments.

b) Biliary Excretion of ICG. Under the same conditions as described under a), biliary excretion of ICG during infusion of saline, dehydrocholate and taurocholate was investigated. Table 1 shows the results of the ICG excretion and bile production after ICG injection of 3.9 and 12.9 μmoles/kg. When an injection of 12.9 μmoles ICG/kg was given, taurocholate caused the most pronounced stimulation of ICG excretion: 138% (mean value of 6 periods of 10 min after injection). During the same collection time dehydrocholate administration enhanced biliary output of ICG to a smaller extend: 55% during the infusion of 106 μmoles/h and 45% during the infusion of 268 μmoles/h.

Although the choleresis produced by taurocholate (stimulation 195%) was much smaller compared with that caused by an equimolar infusion of dehydrocholate (stimulation 297%), the stimulation of biliary ICG excretion was higher, indicating that the increase in biliary excretion was not related to bile flow only. In agreement with this, it can be seen that the increase in choleresis produced by the higher infusion of dehydrocholate did not lead to a greater stimulation of the ICG excretion. (Table 1).

A different situation occurred when a dose of 3.9 μmoles/kg ICG was tested. Here the infusion of taurocholate had no influence at all on biliary ICG excretion (Table 1). An equimolar amount of dehydrocholate, the lower concentration 106 μmoles/h, caused a distinct increase in biliary output of ICG (56%). On the contrary, the higher dehydrocholate infusion (268 μmoles/h) resulted in a slight but significant decrease in the excretion of ICG (31%).

Discussion

Various pharmacokinetic models have been proposed for the hepatic elimination of drugs. Many studies have been based on plasma levels of BSP. The blood level of this dye may be described approximately by a bi-exponential equation as a function of time, with a rapid and a slower component. The simplest explanation for the disappearance pattern is a two compartment system with "run-off". (Richards, 1965). The primary rapid decline in plasma concentration is assumed to be due to a fast hepatic uptake and the slow component reflects the biliary excretion process after a steady-state is reached between the liver and the plasma compartments. When the storage capacity for a certain substance is high, it will take a long time to establish a steady-state between plasma and liver and the second component is initiated at a low plasma concentration. This is probably the case with ICG and consequently a mono-exponential

decline is found. A second component in the plasma curve which is below the detectable level could exist, because the concentrations after 12 min were higher than the blank value, but too low to be reliably detected. The appearance of a second component in the plasma disappearance curve of ICG at very low levels has been described by Hunton *et al.* (1960) and Hargreaves (1968).

During dehydrocholate infusion, the half life of the first phase (2.2 min) was similar to the half life in the control experiment, indicating that the primary uptake of ICG in the liver is not affected by this bile salt.

Paumgartner *et al.* (1969) reported that the bile salt glycocholate did not affect the uptake of ICG in the liver. Kelman-Sraer *et al.* (1973) found that in rabbits plasma disappearance of rose bengal is retarted by dehydrocholate.

Under the influence of a dehydrocholate infusion a distinct second component in the plasma curve occurs with a half life of 13.5 min (106 μmoles/h) and 15.0 min (268 μmoles/h). In the simplified pharmacokinetic scheme mentioned above, the appearance of this second component may be explained in two ways:

a) by a diminution in the storage capacity of the liver,

b) by inhibition of the biliary excretion.

Inhibiton of the biliary excretion is not likely because during dehydrocholate infusion a stimulation of ICG excretion is found. (Table 1). Therefore a diminished storage capacity for ICG during dehydrocholate infusion seems to be more probable.

Competition of dehydrocholate with ICG for intracellular binding to specific proteins could lead to a quicker establishment of the steady-state between the liver and the plasma compartments. This would be in agreement with the finding that the shift of the second phase to higher plasma levels of ICG, under the influence of dehydrocholate, is dependent on the dose of dehydrocholate. Andrews and Richards (1960) have found that taurocholate and dehydrocholate interfere with the binding of BSP by proteins in hepatic cytosol; the same might apply to the ICG binding by these protein.

Levi *et al.* (1969) showed that, after injection in vivo, ICG is bound to the socalled X and Y proteins of rat liver supernatant; BSP on the other hand is mostly bound to Y and Z proteins. They found that taurocholate interferes with BSP binding to Z proteins only at very high concentration of the bile salt. No data on the influence of taurocholic acid or dehydrocholic acid on ICG binding to these proteins are available. Moreover it is not known to what extent the size of the X and Y protein pool accounts for the storage capacity for ICG. The lack of effect of taurocholate on the plasma disappearance curve of ICG might be due to the smaller affinity of taurocholate for the presumed ICG binding sites or to a lower concen-

tration of taurocholate at the competion sites compared with dehydrocholate or to a highly stimulated biliary ICG excretion under the influence of taurocholate. However from this study we cannot draw any further conclusions.

The effects of the bile salts on the biliary ICG excretion are much more difficult to explain. Pharmacokinetic interactions of drugs are difficult to interpret because interactions at various sites are possible. Regarding the effect of the choleretics on the biliary output of ICG, an interplay of at least four possible interactions should be considered:

1. When the limiting factor on the maximal transfer of ICG from the liver into bile is the concentration of ICG in canalicular bile, an increased canalicular bile flow rate would lead to an increased transport of ICG.

2. During the administration of micelle-forming bile salts, biliary excretion of substances apt to be taken up in biliary micelles (Bickel and Minder, 1970) might be stimulated.

3. Competition for excretion between ICG and the bile salt at canalicular sites. The outcome of this interaction depends both on the concentrations of the substances at the carrier sites and on the affinity for these carrier sites.

4. Competition for binding sites in the liver.

This implies that the final effect of choleretics on the biliary output of ICG might be the sum of stimulatory and inhibitory interactions. Moreover it is known that ICG inhibits the forming of the bile salt-independent fraction. (Horak *et al.*, 1973).

Our results show that, at a dose of 12.9 μmoles/kg ICG, the biliary excretion of ICG is considerably stimulated by both taurocholate and dehydrocholate. The effect of taurocholate is much more pronounced than that of dehydrocholate in spite of the fact that dehydrocholate gives a much higher choleretic response. Thus, excretion rate cannot be determined by bile flow alone. According to the consideration mentioned above, the difference between taurocholate and dehydrocholate may be explained in two ways:

1. The pronounced effect of taurocholate is related to its micelle forming ability.

2. Compared with taurocholate, dehydrocholate has a higher affinity (Ks) for the transport sites and/or reaches higher concentrations (S/K_s) in the vicinity of these sites.

The presence of competition between bile salts and ICG for the carrier sites is further supported by the fact that an increased dehydrocholate dose (268 μmoles/h) does not result in a further increase in ICG excretion. It was not possible to find a similar effect with taurocholate because higher concentrations of taurocholate were lethal.

Furthermore at the dose of 3.9 μmoles/kg ICG, the situation in which the competition for bile salts is more favourable, taurocholate has no influence and dehydrocholate (268 μmoles) even has a negative effect on the biliary output of the dye.

At this moment the available information does not allow definite conclusions and further studies with subcellular components will be necessary to elucidate the possible interactions.

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Choleresis and Hepatic Transport Mechanisms*

II. Influence of Bile Salt Choleresis and Biliary Micelle Binding on Biliary Excretion of Various Organic Anions

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Summary. To investigate, whether binding to micelles has a function in hepatic transport, biliary excretion of three organic anions, phenolphthalein-β-*D*-glucuronide (PG), dibromosulphthalein (DBSP) and indocyanine green (ICG) was studied in rats during saline, taurocholate or dehydrocholate administration. Taurocholate causes a weak choleresis with formation of biliary micelles, dehydrocholate a strong choleresis with little micelle formation. The two bile salts did not uniformly influence biliary excretion of the organic anions: biliary excretion of ICG (12.9 μmoles/kg) and DBSP (75.0 μmoles/kg) was stimulated by both bile salts: ICG excretion most pronounced by taurocholate and DBSP excretion most strongly by dehydrocholate. Biliary output of PG (25.8 and 200 μmoles/kg) was not stimulated by bile salt administration. Binding of PG, DBSP and ICG to biliary micelles was studied in sedimentation experiments by ultracentrifugation. PG, DBSP and ICG in bile showed a similar sedimentation pattern as ^{3}H-taurocholate in bile, which indicates an association of all three anions with biliary micelles.

Thus, the influence of bile salts on biliary transport of organic anions varies with the compound studied and the bile salt used, effects which cannot be explained by differences in binding to biliary micelles.

Key words: Biliary Excretion — Choleresis — Organic Anions — Bile Acids.

The influence of choleresis on biliary excretion of drugs has been subject of many studies. Most authors agree that there is not a simple relation between stimulation of bile flow and stimulation of the biliary output of organic anions. Taurocholate, which promotes forming of biliary micelles (Sperber, 1965), stimulates biliary excretion of certain compounds more than agents which have been suggested to induce choleresis with little micelle formation like theophylline (Erlinger and Dumont, 1973a), dehydrocholate (Hardison, 1971), 4-methylumbelliferone, SC 2644 (Cook *et al.*, 1954), hydrocortisone (Macarol *et al.*, 1970). Therefore it was suggested, that binding to micelles may have a function in biliary ex-

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* The previously published paper of Vonk *et al.* (1974) is regarded as paper I in this series.

cretion of drugs (Hardison and Apter, 1972; Billing *et al.*, 1973; Gibson and Forker, 1974; Vonk *et al.*, 1974), though there may be more sites of interaction between the choleretic agent and the drug during hepatic transport. In most of these studies BSP was chosen as a representative of the organic anions. However, BSP is conjugated to a high extent with glutathione, which might complicate the interpretation of the interaction data. Therefore, we have investigated the influence of choleresis on biliary excretion of some other organic anions, which are not metabolized: phenolphthalein-β-D-glucuronide (PG) (Millburn *et al.*, 1967; Mulder, 1973), indocyanine green (ICG) (Cherrick *et al.*, 1960; Klaassen and Plaa 1969) and dibromosulphthalein (DBSP) (Javitt, 1964). These compounds are taken up by the liver and excreted in bile to a large extent.

The present investigations were concerned with the influence of bile salt choleresis on the biliary excretion of ICG, PG and DBSP and a possible role of micelles in this excretory process.

Materials and Methods

Chemicals. The chemicals used were obtained from the following sources: ICG, DBSP: Hynson, Westcott and Dunning Inc., Baltimore, U.S.A. PG: Sigma Chem. Comp., St. Louis, U.S.A. sodium dehydrocholate and sodium taurocholate: Fluka A.G., Buchs, Switzerland.

^{3}H-taurocholic-acid: New England Nuclear Inc., Boston, U.S.A.

PG, ICG and DBSP were dissolved in distilled water, the other chemicals in saline.

Chemical Analysis. PG was determined by the method described by Mulder (1973), ICG according to the method described previously (Vonk *et al.*, 1974). DBSP was diluted with 0.1 N NaOH and measured spectrophotometrically at 580 nm. Radiochemical analyses were performed according to Meijer *et al.* (1972).

Procedure in Animals. Male Wistar rats (280—320 g), which had free access to food and water, were anesthesized by an intraperitoneal injection of sodiumpentobarbital (Nembutal®) (60 mg/kg) and artificially respirated during the experiments. After midline incision the renal pedicles were ligated and the bile duct was cannulated with polyethylene tubing. The body temperature, measured rectally, was kept between 37.5 and 38.0° C by means of a heating lamp. Infusion (3.8 ml/h) were given via the jugular vein by means of a Braun (Melsungen, Germany) constant infusion pump. Infusions were started when the temperature of 37.5° C was established. Constant infusions were preceded by a priming dose of 0.5 ml of the infusion solution; in this way 30 min after starting the infusion, bile production was fairly constant. At that moment a single injection of the dye was given via the same jugular vein. In some experiments blood pressure was measured in the carotid artery in order to check the general condition. Injection of the substances according to the procedure mentioned above, only slightly affected blood pressure during a few minutes.

Sedimentation of Biliary Micelles. Biliary micelles can be sedimented by ultracentrifugation (Bickel and Minder, 1970). To study the association of organic anions with biliary micelles, bile and bile salt solutions containing the substances under study, were subjected to centrifugation in a Spinco ultracentrifuge. Centrifugation was performed in the Ti 50 rotor with an adaptor at 45000 RPM, (140000 g_{av}) for

210 min at 20°C. In one type of experiments biliary micelles were labeled with ^{3}H-taurocholate, which was infused *in vivo* according to the procedure mentioned above. Bile was collected during up to 1 and 2 hrs after the injection of the compounds. For *in vitro* studies blank bile was obtained by collection during 2 hrs. Sedimentation of the organic anions was studied in 5 different solutions:

a) bile containing the substances after their injection *in vivo* in a concentration of: ICG 0.6 mM, DBSP 6.5 mM and PG 8.5. mM,

b) bile containing the substances after their injection *in vivo* during a simultaneous constant infusion of ^{3}H-taurocholate according to the procedure mentioned above,

c) bile, in which such an amount of the substance was dissolved, that the concentration of the *in vivo* situation was reached,

d) aqueous taurocholate solution (53 mM, which is in the range of the physiological concentration), in which such an amount of the substance was dissolved, that the concentration of the *in vivo* situation was reached,

e) aqueous dehydrocholate solution (35 mM, a concentration of dehydrocholate, which is reached in bile assuming all dehydrocholate is excreted in bile during an infusion of 106 μmoles/h), in which the substance was dissolved to a concentration equal to the *in vivo* situation.

The sedimentation pattern of ^{3}H-taurocholate in bile is assumed to reflect the sedimentation pattern of biliary micelles, as it is known, that the major part of taurocholate in bile is associated with other bile salts, cholesterol and phospholipids to form macromolecular aggregates (Carey and Small, 1972). After centrifugation different fractions were obtained by pipetting from the tube.

Determination of Biliary Transport Maximum (T_m). An injection of the organic anion was given to the animal and the maximal biliary excretion rate was measured. The biliary transport maximum (T_m, in μmoles/min/kg b.w.) is assumed to be reached, when the maximal biliary excretion rate does not increase, when a higher dose of the organic anion is injected. In this case the biliary excretion rate is about the same in the second, third and fourth period after injection of DBSP and in the second and third period after injection of PG. With ICG no T_m was determined; ICG was given in a rather low dose (12.9 μmoles/kg), because of the inhibitory effects of ICG on bile production (Horak *et al.*, 1973).

Statistical Analysis. Statistical comparisons were made using Wilcoxon's test. Unless specified otherwise, the term significant means a P value of less than 0.05.

Results

A. Effect of Bile Salt Choleresis on Biliary Excretion of PG, DBSP and ICG

In order to produce choleresis, an infusion of taurocholate (106 μmoles/h) and dehydrocholate (106 μmoles/h) was given, while an infusion of saline served as the control. Mean bile production in a steady state was 60 μl/min/kg b.w. during saline, 100 μl/min/kg b.w. during tarocholate administration and 150 μl/min/kg b.w. during dehydrocholate infusion. The effect of this choleresis on the biliary excretion of PG, DBSP and ICG was examined.

Two doses of PG were used: 25.8 and 200 μmoles/kg. The results are shown in Figs. 1 and 2, respectively. With the lower dose of PG, biliary excretion of the substance was not significantly changed during bile salt

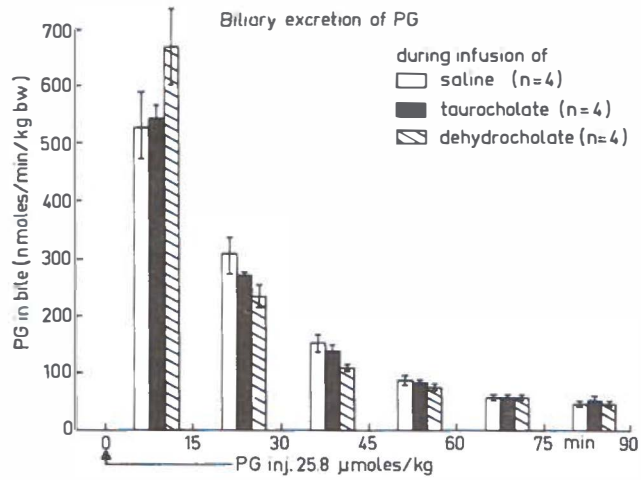

Fig. 1. Biliary excretion of PG during 90 min after i.v. injection of 25.8 µmoles/kg b.w. during infusion of saline, taurocholate (106 µmoles/h) and dehydrocholate (106 µmoles/h). Mean values ± S.E.M. * Significantly deviating from controls

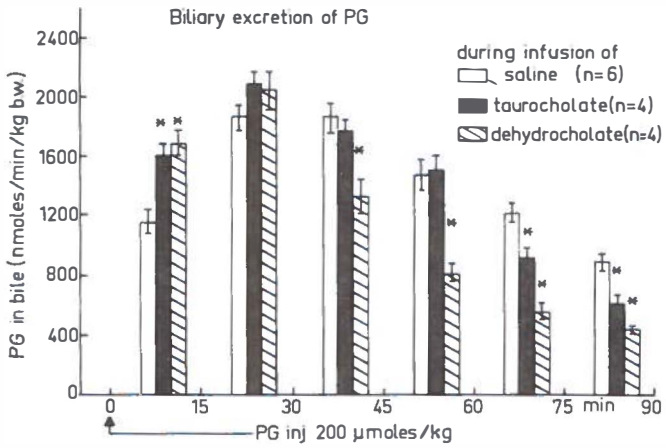

Fig. 2. Biliary excretion of PG during 90 min after i.v. injection of 200 µmoles/kg b.w. during infusion of saline, taurocholate (106 µmoles/h) and dehydrocholate (106 µmoles/h). Mean values ± S.E.M. * Significantly deviating from controls

administration. At the close of 200 µmoles PG/kg, T_m of PG is reached. Maximal biliary excretion rate was calculated to be about 1.9 µmoles/min/kg b.w. at a maximal biliary concentration of 23 mM, which is in accordance with data presented by Mulder (1973). At the dose of 200 µmoles/kg PG excretion was stimulated during bile salt infusion in the first period, which is possibly due to a decrease of the biliary dead space transit time, caused by the choleresis. In all other periods no stim-

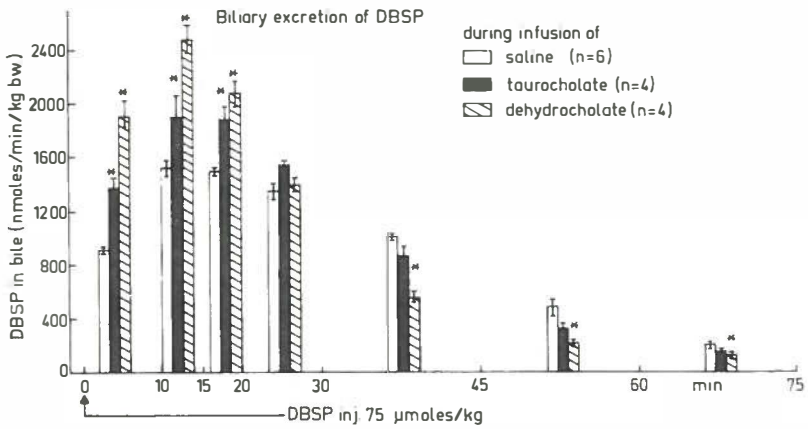

Fig. 3. Biliary excretion of DBSP during 75 min after i.v. injection of 75 µmoles DBSP/kg b.w. during infusion of saline, taurocholate (106 µmoles/h), and dehydrocholate (106 µmoles/h). Mean values ± S.E.M. * Significantly deviating from controls

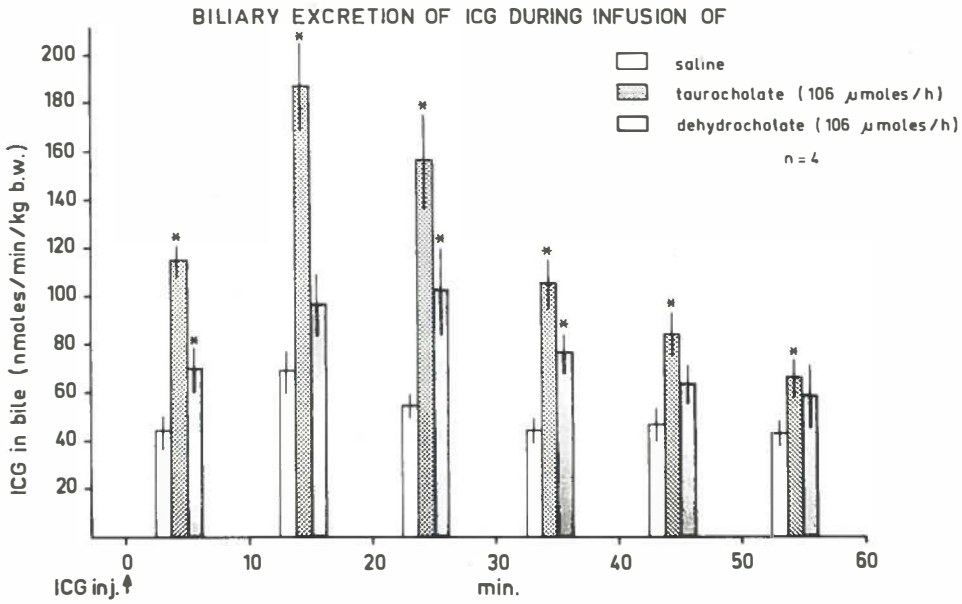

Fig. 4. Biliary excretion of ICG during 60 min after injection of 12.9 µmoles ICG/kg b.w. during infusion of saline, taurocholate (106 µmoles/h) and dehydrocholate (106 µmoles/h). Mean values ± S.E.M. * Significantly deviating from controls

ulation of PG excretion could be found: biliary output of PG is even depressed in the latter four periods during dehydrocholate administration and in the latter 2 periods during taurocholate administration.

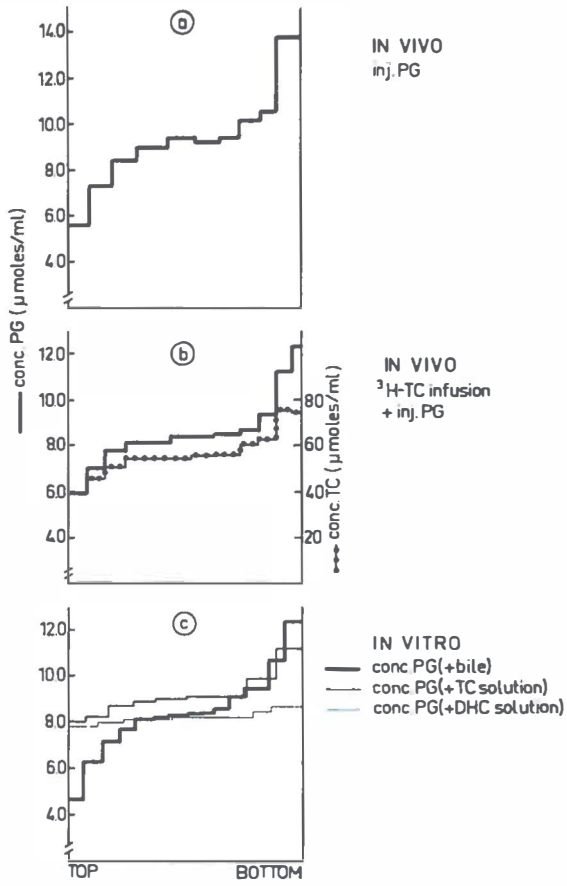

Fig. 5a—c. Sedimentation pattern after centrifugation of: (a) bile collected after injection of PG (100 μmoles/kg) during 90 min; (b) bile collected after injection of PG (100 μmoles/kg) during 75 min, while ^{3}H-taurocholate was administrated (106 μmoles/h); (c) PG added to bile and solutions of taurocholate (53 mM) and dehydrocholate (35 mM)

The maximal biliary excretion rate for DBSP was 1.9 μmoles/min/kg b. w. and maximal biliary conc. was 22 mM. The observed DBSP T_m value exceeds that found by Klaassen and Plaa (1968) (1.2 μmoles/min/kg b. w.). The influence of bile salt choleresis on biliary excretion of DBSP is illustrated in Fig. 3. A stimulation of biliary output is found during bile salt administration, most pronounced in the first 20 min during dehydrocholate administration.

Fig. 4 shows the biliary excretion of ICG. Taurocholate stimulates ICG output markedly in all periods examined; the influence of dehydrocholate administration was much less pronounced and only statistically significant in some of the periods.

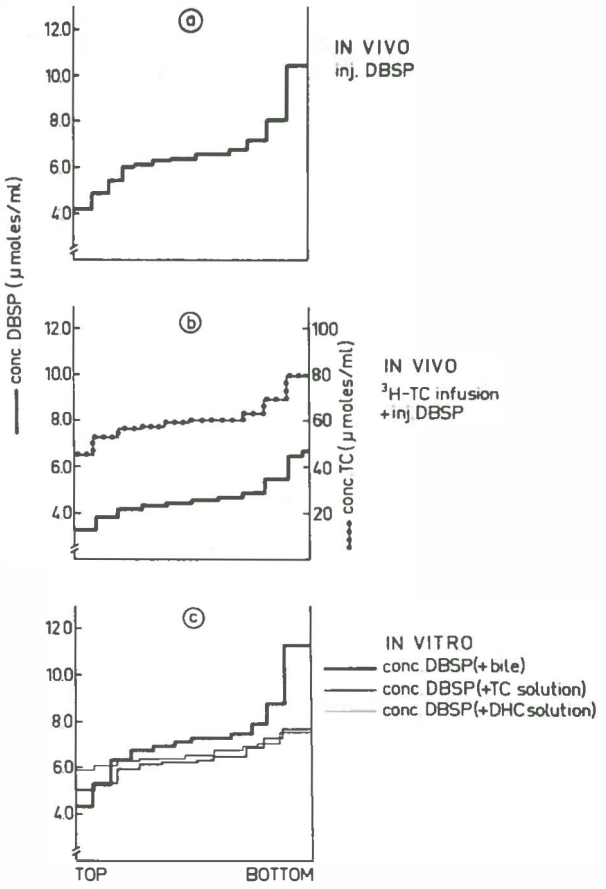

Fig. 6a—c. Sedimentation pattern after centrifugation of: (a) bile collected after injection of DBSP (50 μmoles/kg) during 90 min; (b) bile collected after injection of DBSP (50 μmoles/kg) during 75 min, while ^{3}H-taurocholate was administered (106 μmoles/h); (c) DBSP added to bile and solutions of taurocholate (53 mM) and dehydrocholate (35 mM)

The results indicate that the influence of bile salt administration on biliary output of organic anions varies with the compounds used: DBSP and ICG excretion is generally stimulated: PG excretion, however, is not affected and in some cases slightly depressed.

B. Sedimentation of Bile With PG, DBSP and ICG in vivo *and* in vitro

Bile and aqueous solutions of dehydrocholate and taurocholate containing PG, DBSP and ICG were centrifuged as described under materials and methods. Figs. 5—7 show the sedimentation patterns after centrifugation of PG, DBSP and ICG. The panels "a" represent the sedimentation patterns of the organic anions in bile, obtained after their

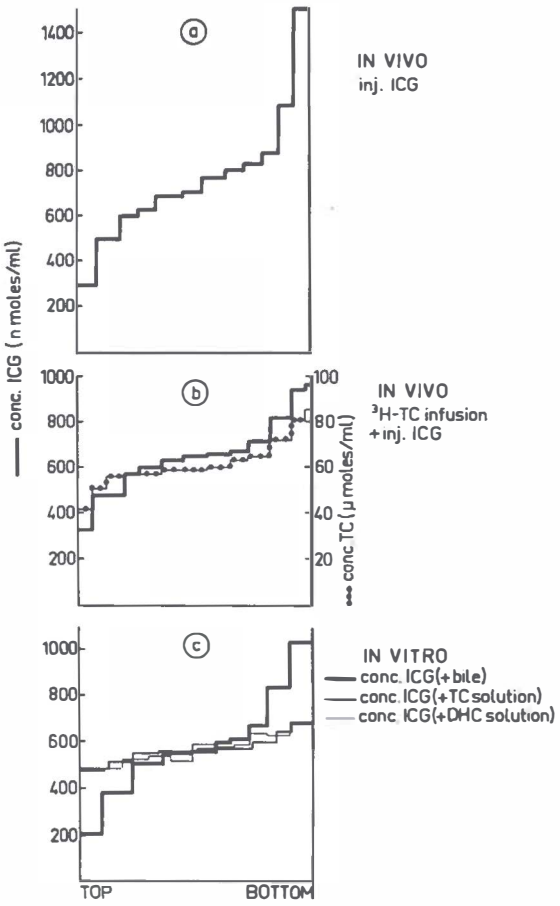

Fig. 7a—c. Sedimentation pattern after centrifugation of: (a) bile collected after injection of ICG (12.9 μmoles/kg) during 120 min; bile collected after injection of ICG (12.9 μmoles/kg) during 90 min, while ^{3}H-taurocholate was administered (106 μmoles/h); (c) ICG added to bile and solutions of taurocholate (53 mM) and dehydrocholate (35 mM)

injection *in vivo*. Panels b show the sedimentation pattern of the organic anions in bile, obtained after their injection *in vivo* during a simultaneous infusion of ^{3}H-taurocholate. In this case also the sedimentation pattern of ^{3}H-taurocholate is measured. The sedimentation pattern of all three organic anions resembles that of taurocholate. Distribution of taurocholate is assumed to indicate the position of the biliary micelles in the tubes. For all three compounds, the concentration differences along the centrifugation tube were more pronounced in normal bile than in bile obtained during taurocholate infusion. The panels c represent the sedimentation pattern after centrifugation of an aqueous taurocholate solution, an

aqueous dehydrocholate solution and normal bile to which PG, DBSP or ICG was added. Almost no sedimentation occurred in dehydrocholate solutions and slight sedimentation occurred in the aqueous taurocholate solution. Ultra centrifugation of bile, to which the compounds were added *in vitro* and bile, obtained after injection of the organic anions *in vivo*, resulted in very similar sedimentation patterns.

Discussion

The study of the influence of choleresis on biliary excretion of organic compounds might be helpful in elucidating the mechanism of the biliary transport process. One of the most extensively studied compounds is bromosulphthalein (BSP). An increase in BSP T_m observed during bile salt choleresis has been described in the intact rat (Dhumeaux *et al.*, 1970), the isolated rat liver perfusion (Boyer *et al.*, 1970), the dog (O'Maille *et al.*, 1966; Ritt and Combes, 1967), and the sheep (Gronwall and Cornelius, 1970). Morris (1972), found that taurocholate elevates BSP T_m in dogs, whereas theophylline choleresis had no effect. Other authors (Erlinger and Dumont, 1973 b; Barnhart and Combes, 1974; Gibson and Forker, 1974) also observed differences between the effect of taurocholate and other non-bile salt choleretics on BSP T_m. From these experiments it became evident, that stimulation of bile flow per se does not necessarily cause an increase in biliary excretion rate of BSP. Possibly an additional effect of taurocholate plays a role in this phenomenon.

Taurocholate administration also increases biliary output of conjugates of iopanoic acid (contrast agent in cholecystography) (Berk *et al.*, 1974), thyroxine (Hillier, 1974) and bilirubin (Goresky *et al.*, 1974), (mainly glucuronides); however, interactions at the level of biotransformation in these studies can not be excluded.

It is uncertain whether the stimulatory action found with taurocholate does also apply to other bile salts. Gibson and Forker (1974) could not find a reproducible increase of BSP T_m with dehydrocholate in dogs, and Bloomer *et al.* (1973) found an inhibition of bilirubin excretion in man during dehydrocholate choleresis. However, Berk *et al.* (1974) showed that both dehydrocholate and taurocholate stimulate biliary excretion of iopanoic acid conjugates, and O'Maille *et al.* (1966) described a stimulatory effect of taurocholate, but also of dehydrocholate and glycocholate on BSP T_m.

Furthermore the question arises, whether results obtained with BSP are also applicable to other organic anions. As BSP is metabolically altered during hepatic transport, and as T_m and choleretic properties for the parent compound and the glutathion-conjugate (BSP-GSH) have recently been shown to be different (Varga *et al.*, 1974; Schulze and Czok,

1974), separate determination of BSP and BSP-GSH is necessary to exclude complications due to influences on the conjugation reaction.

To exclude influences of biotransformation, we have performed our experiments with DBSP, ICG and PG. The organic anions were given by single injection (see Materials and Methods). The present study indicates, that the influence of bile salt administration on the biliary output of organic anions does not only depend on the particular bile salt used, but also on the organic anion studied. In the case of ICG and DBSP with both bile salts, a stimulation is found. PG excretion is not affected at all by both bile salts. In fact a significant inhibition is found during dehydrocholate infusion. This inhibition might be due to competition between dehydrocholate and PG for a transport carrier or to non-specific effects of dehydrocholate. In the case of competition between PG and dehydrocholate, inhibition of PG excretion would be expected to be more pronounced at a lower dose of PG. The results of Fig. 1 do not support this idea. Our results are in contrast with those of other studies, where a stimulation of the biliary excretion of glucuronides by bile salt choleresis was found (Berk *et al.*, 1974; Goresky *et al.*, 1974; Hillier, 1974).

The following mechanisms have been proposed in order to explain an observed stimulation of biliary excretion of organic anions.

1. Facilitation of the transport from liver into bile by allosteric interactions at the supposed carrier sites in the canalicular membranes. This model was proposed by Forker and Gibson (1973) to explain an observed increase of BSP T_m by taurocholate. This hypothesis was also put forward by Berk *et al.* (1974) explaining the interaction of iopanoic acid and bile salts.

2. Stimulation of micelle formation inside the liver cell (Desai *et al.*, 1965; Swell *et al.*, 1968), binding of the anions to the aggregates and subsequent exocytoses of these aggregates into bile canaliculi.

3. Binding of anions to micelles in the canaliculi with consequent lowering of the free concentration of the drug leading to diminished transfer of the drug from bile back into the liver. This would especially influence net biliary transport at high concentrations, when back diffusion may be relatively important.

Taurocholate is able to form micelles (Sperber, 1965). Infusion of taurocholate has been shown to increase biliary output of phospholipids and cholesterol (Hardison and Apter, 1972; Swell *et al.*, 1968), thus it seems reasonable to assume, that formation of mixed biliary micelles is stimulated. Dehydrocholate or its metabolites (Gérolami *et al.*, 1972; Soloway *et al.*, 1973; Desjeux *et al.*, 1973) have a low tendency to form micelles and stimulate biliary excretion of phospholipids and cholesterol only very little (Hardison, 1971; Wheeler and King, 1972).

Our studies do not provide supporting evidence for a general allosteric-facilitated membrane transport, because biliary excretion of PG is not stimulated. Assuming allosteric interaction, it is difficult to imagine, that taurocholate would stimulate only DBSP and ICG, but not PG.

Both, the second and third mechanism assume binding to micelles, a phenomenon, which could be experimentally confirmed in the present investigation. We used ultracentrifugation studies to examine binding of organic anions to biliary micelles, because ICG, which easily forms aggregates, cannot pass dialysis membranes. The sedimentation patterns of the micelles are likely reflected in the ^{3}H-taurocholate gradient. Ultracentrifugation of an aqueous taurocholate solution resulted in only slight concentration differences, while taurocholate in bile showed a very distinct sedimentation, which supports the assumption, that taurocholate is a marker of biliary micelles. Our study shows no differences in binding to biliary micelles of the organic anions. Although the nature of the binding to and/or incorporation into the biliary micelles remains to be investigated, it is clear that binding occurs readily, because extensive sedimentation of the organic anions in bile was observed after addition of the substances *in vitro*. We concluded, that the unequal stimulation of the organic anions by taurocholate cannot be explained by differences in micelle binding and evidently other factors must play a role. A facilitatory role in net biliary transport by binding to micelles in the hepatocytes and/or preventing reflux from bile to liver by binding in the canaliculi as a sole explanation seems to be unlikely. The result of the present investigation further emphasizes, that the influence of bile salts on biliary transport of organic anions depends on the bile salt used and varies with the organic anion studied.

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Choleresis and Hepatic Transport Mechanisms

III. Binding of Ouabain and K-Strophanthoside to Biliary Micelles and Influence of Choleresis on Their Biliary Excretion

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Summary. To investigate binding of drugs to biliary micelles as a possible factor in the hepatic transport process, interaction of two uncharged compounds, ^{3}H-ouabain and ^{3}H-K-strophanthoside with biliary micelles was studied by ultracentrifugation of bile. The various bile acids normally present in rat bile were predominantly associated with cholesterol containing micelles, but not to the same extent. The tendency of the bile salts to be associated with mixed micelles was the greatest for conjugated chenodeoxycholate, somewhat lower for conjugated deoxycholate and the least for conjugated cholate. The sedimentation patterns of the water-soluble cardiac glycosides, added in vitro, indicated binding to mixed biliary micelles as well as non-cholesterol containing micelles. Also mannitol, a drug used to estimate canalicular bile flow, was found to be associated with both categories of biliary micelles.

In spite of the binding of cardiac glycosides to the micelles, administration of taurocholate, which promotes formation of biliary micelles, did not stimulate biliary output of both glycosides. Also administration of the choleretics dehydrocholate and ethacrynic acid failed to enhance biliary output of the glycosides.

These results indicate, that binding of drugs to biliary micelles diminishes the free concentration of drugs in bile and confirms earlier studies with organic anions that binding to biliary micelles is not a pertinent factor in the rate of biliary excretion.

Key words: Biliary excretion – Cardiac glycosides – Choleresis – Bile acids – Bile micelles.

INTRODUCTION

The mechanism by which bile acids influence hepatic transport of drugs still is unknown. Most studies in this field are dealing with the influence of choleretic agents on the hepatic transport of organic anions. In 1966 O'Maille observed that infusion of bile acids stimulates biliary excretion of BSP. Subsequently, several authors described a stimulatory effect of bile acids on biliary output of organic anions (Ritt and Combes, 1967; Boyer et al., 1970; Forker and Gibson, 1973; Vonk et al., 1975). Several factors have been proposed to be responsible for this stimulating effect of bile salts: (a) an increased bile flow (O'Maille et al., 1966); (b) modification of the transport carriers in the canalicular membranes (Forker and Gibson, 1973); (c) an increased number of hepatocytes, participating in biliary transport of drugs (Goresky, 1975); (d) an enhanced biliary micelle formation stimulating biliary output of those drugs, which are included in biliary micelles (Billing et al., 1973; Gibson and Forker, 1974).

This study is concerned with the influence of choleretic agents on hepatic transport of 2 uncharged compounds, ouabain and K-strophanthoside in relation to the binding of both compounds to biliary micelles.

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MATERIALS AND METHODS

Chemicals. The chemicals used were obtained from the following sources: ^{3}H-ouabain and ^{3}H-K-strophanthoside: New England Nuclear Corp. Dreieichenhain, Germany; ^{14}C-Mannitol: Radiochemical Centre Amersham, England; ouabain (g-strophanthin) and K-strophanthoside: Merck AG, Darmstadt, Germany; dehydrocholate (sodium) and taurocholate (sodium): Fluka AG, Buchs, Switzerland; ethacrynic acid was a gift from Merck, Sharp & Dohme, Haarlem, The Netherlands.

Chemicals injected into the animals were dissolved in saline before administration.

Radiochemical Analysis. ^{3}H-ouabain, ^{3}H-K-strophanthoside and ^{14}C-mannitol were estimated with a liquid scintillation spectrometer (Nuclear Chicago Mark II). Plasma and bile samples were dissolved in 10 ml of aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.). The samples were counted at an efficiency of about 85%.

for ^{14}C and 35% in case of ^{3}H. Quenching of each sample was corrected for by external standardization. Analysis by thin layer chromatography (Silicagel, n-butanol-H_2O-methanol; 70:10:20) indicated that K-strophanthoside was excreted in bile in the unchanged form for more than 90%, which is in agreement with the results of Marzo et al. (1974). Ouabain is excreted in bile in the unchanged form (Dutta et al., 1963; Kupferberg and Schanker, 1968).

Determination of Bile Salts and Cholesterol. Bile salts and cholesterol were determined by gaschromatography according to the methods described by Roovers et al. (1968) and Van Berge Henegouwen et al. (1974) with minor modifications. After methylation the bile acids were chromatographed as their trifluoroacetate derivatives on a column of 1% OV-210 on Cromosorb W-HP 100–120 mesh and analysed by flame ionization in a Packard-Becker Model 419 gaschromatograph.

Procedure in animals was performed according to Vonk et al. (1975). Blood samples of about 150 µl were taken from the carotid artery and collected in heparinized capillary tubes (Sherwood, Med. Ind. Inc., St. Louis, U.S.A.). The substances were injected over a period of 15 s to avoid a severe decrease of blood pressure.

Statistical Analysis. Statistical comparisons were made using Wilcoxon test. Unless specified otherwise, the term significant means a P value of less than 0.05.

RESULTS AND DISCUSSION

To sediment biliary micelles, bile was centrifugated at 162000 g_{av} for 17 h or 45 h at 20° C in a Beckman L2-65B ultracentrifuge (Rotor Ti 50). Centrifugation time was considerably longer than in our previous study (Vonk et al., 1975), which allows not only a qualitative, but also a quantitative estimation of binding to cholesterol containing micelles. After centrifugation, the various fractions in the tube were separated by pipetting and subjected to gaschromatographic analysis to control sedimentation of bile acids and cholesterol. Figure 1 a and b represents the sedimentation pattern of bile acids and cholesterol after 17 h and 45 h of centrifugation respectively. The absence of cholesterol in the top fraction after centrifugation indicates absence of cholesterol containing micelles (mixed micelles) in that fraction. The concentration of bile acids in this fraction therefore is regarded as the "free" bile acid concentration. The percentage of the substances bound to biliary micelles then could be calculated: $\frac{C_u - C_f}{C_u} \times 100\%$, assuming that the free drug concentration in all fractions is the same. C_u = concentration of the compound in the tube at the beginning of the experiment; C_f = concentration of the compound in the upper fraction of the tube after centrifugation. The "free" concentrations and the percentages incorporated into biliary micelles of some compounds naturally present in rat bile, after 17 h and 45 h centrifugation, are presented in Table 1. In spite of a complete removal of mixed micelles from the upper fraction after 17 h of centrifugation, the "free" concentration of the various bile acids further decreased when the centrifugation time was prolonged to 45 h. This may indicate a slower sedimentation of the small non-cholesterol containing micelles compared with the sedimentation of cholesterol containing micelles with higher seize and/or density. All conjugated bile acids were partly incorporated in mixed biliary micelles. Conjugated chenodeoxycholate had a higher tendency to be incorporated than conjugated cholate or conjugated deoxycholate (Table 1). Also conjugated lithocholate was associated with mixed micelles, but exact determination was not possible because of the low concentration of lithocholate in normal rat bile. The same applies to unconjugated bile salts, of which the biliary concentration was very low. Other constituents of the micelles, lecithin, bilirubin or lipoproteins (Verschure and Mijnlieff, 1956; Juniper, 1965) were not investigated. The "free" concentration of conjugated cholate, measured with this technique was 4.4 ± 0.4 mM and 2.8 ± 0.1 mM, determined after 17 h and 45 h of centrifugation respectively. The critical micellar concentration of taurocholate at 20° C in 150 mM NaCl is 3.2 mM (Carey and Small, 1972). In vitro addition of cardiac glycosides to bile did not change the "free" concentration of the bile acids.

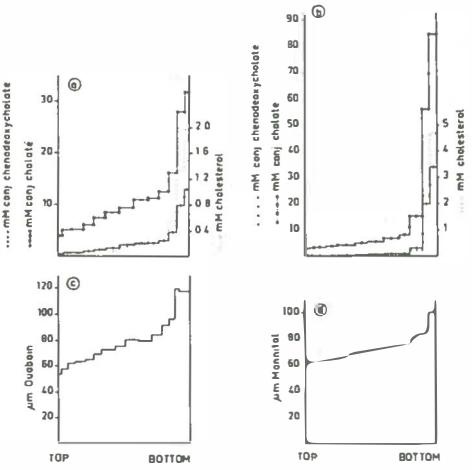

Fig. 1. Sedimentation pattern of (a) bile salts and cholesterol after 17 h of centrifugation of normal bile from female rats; (b) bile salts and cholesterol after 45 h of centrifugation of normal bile from female rats; (c) ^{3}H-ouabain after 17 h of centrifugation of bile from female rats, to which ouabain was added in vitro (78 µM); (d) ^{14}C-mannitol after 17 h of centrifugation of bile from female rats, to which mannitol was added in vitro (71 µM).

Binding of some drugs to mixed biliary micelles was subsequently measured. The compounds were added to bile in vitro and after centrifugation the

Table 1. Data concerning incorporation into biliary micelles and "free" concentration of some compounds naturally present in rat bile, after 17 h and 45 h of ultracentrifugation (mean values of five experiments) and binding to biliary micelles and "free" concentration of some exogenous compounds after 17 h and 45 h of centrifugation (mean values of three experiments)

Compound	Starting conc. (μM)	17 h of centrifugation		45 h of centrifugation	
		"free" conc. (μM)	% bound	"free" conc. (μM)	% bound
Conj. cholate	12100 ± 760	4390 ± 420	63 ± 3	2800 ± 80	76 ± 2
Unconj. cholate	150 ± 50	–	–	–	–
Conj. chenodeoxycholate	2220 ± 130	350 ± 40	87 ± 2	160 ± 20	95 ± 1
Conj. deoxycholate	460 ± 40	90 ± 7	80 ± 3	60 ± 20	87 ± 2
Conj. lithocholate	70 ± 6	< 22	> 65	< 22	> 65
Cholesterol	256 ± 28	< 20	> 94	< 20	> 94
^{3}H-ouabain	78	55 ± 1	29 ± 1	32 ± 1	54 ± 2
^{3}H-K-strophanthoside	53	37 ± 1	29 ± 1	18 ± 1	62 ± 2
^{14}C-mannitol	71	59 ± 1	16 ± 1	52 ± 1	27 ± 1

concentration of the compounds in the various fractions was determined and the free drug concentration and percentage bound to mixed biliary micelles was calculated. ^{3}H-ouabain, ^{3}H-K-strophanthoside and ^{14}C-mannitol were added to bile in vitro to a concentration of 78, 53 and 71 μM respectively, which was in the same range as the concentration occurring in bile after in vivo injection of the drugs (see Fig. 2). Percentage of binding after 17 h and 45 h of centrifugation (Table 1) and two sedimentation patterns after 17 h of centrifugation are shown (Fig. 1c, d). All 3 good water-soluble compounds were partly bound to biliary micelles. It remains to be established, whether this binding means incorporation into the polar shell, the non-polar core or represents some type of inclusion into the micelles. It is also shown that the calculated binding increases with a longer centrifugation time. Since after 17 h of centrifugation cholesterol is absent from the top fraction, further sedimentation of the 3 compounds during longer centrifugation suggests binding of these drugs to the non-cholesterol containing micelles. Also in a taurocholate solution of 12.1 mM this sedimentation could be demonstrated after 45 h of centrifugation. The binding percentage observed after 45 h of centrifugation of bile may be an underestimation of the in vivo binding, because removal of micellar-bound drugs from the upper fraction may still be incomplete. In further experiments we have chosen to restrict the centrifugation time to 17 h, a time in which removal of cholesterol containing micelles from the top of the tube was complete, in order to estimate the *relative* binding of various drugs to biliary micelles.

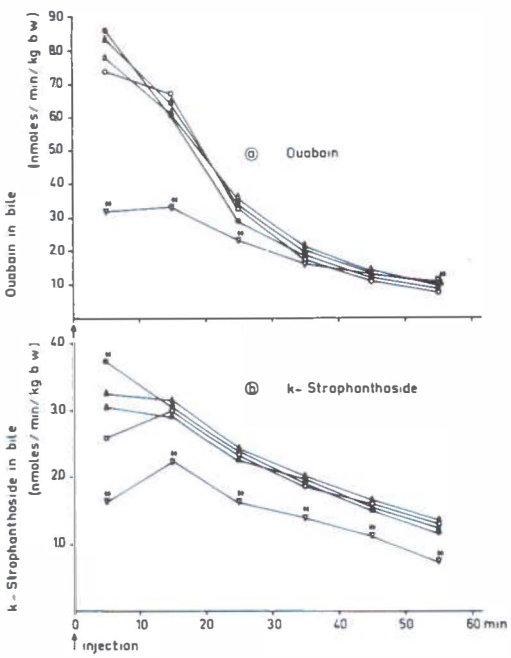

Fig. 2. Biliary excretion rate (nmoles/min/kg b.w.) of (a) ^{3}H-ouabain and (b) ^{3}H-K-strophanthoside after infusion of saline (○——○), taurocholate (106 μmoles/h) (▲——▲), dehydrocholate (106 μmoles/h) (△——△), dehydrocholate (268 μmoles/h) (▽——▽) and ethacrynic acid (63 μmoles/h) (●——●). Mean values of four experiments. Injection of 290 nmoles/kg b.w.

One of the implications of the binding of drugs to biliary micelles is that in calculations of the free drug concentration gradients between plasma-bile or liver-bile, corrections for this micelle binding have to be made, even in case of good water-soluble drugs.

The present results may also have consequences for the estimation of canalicular bile formation using mannitol or erythritol (Forker, 1967). If the erythritol (mannitol) clearance is plotted versus bile flow, in general a linear relationship is found with a slope slightly greater than 1. This may not only be caused by ductular reabsorption of erythritol (mannitol)-free fluid (Forker, 1977), but also by erythritol (mannitol) binding to biliary micelles.

The observed binding of drugs to biliary micelles might also be a factor determining hepatic transport of drugs. Binding to micelles could facilitate biliary excretion. Drugs, which are able to stimulate biliary excretion of micelles, like taurocholate, then would be expected to have a stimulatory effect on hepatic transport of compounds bound to micelles. Figure 2 shows that administration of 106 μmoles/h of taurocholate did not result in a stimulatory effect on biliary excretion of ^{3}H-ouabain and ^{3}H-K-strophanthoside. This indicates that binding of drugs to biliary micelles to the extent as observed with cardiac glycosides, is not a factor regulating biliary output of these drugs. Although the results from the present study as well as those from our previous report on hepatic transport of organic anions (Vonk et al., 1975) indicate, that binding to biliary micelles is not a pertinent factor in the biliary excretion process, it cannot be excluded that bile salts may function in solubilizing some cholephilic compounds like indocyanine green in the bile fluid.

In Figure 2 also the effect of 2 other choleretics, dehydrocholate and ethacrynic acid on biliary excretion of ouabain and K-strophanthoside is shown. None of the choleretics used exerted a stimulatory effect on biliary excretion of the cardiac glycosides, which confirms several earlier reports concerning the influence of choleretics on hepatic transport of ouabain (Kupferberg, 1969; Erttmann and Damm, 1976; Meijer et al., 1976). Plasma disappearance of both cardiac glycosides was not appreciably changed by administration of 106 μmoles/h of bile salts and ethacrynic acid. Administration of 268 μmoles/h of dehydrocholate, however, inhibited significantly plasma-disappearance as well as biliary excretion of the cardiac glycosides. The mechanism of this inhibition is yet unclear (Kupferberg, 1969; Meijer et al., 1976).

This study indicates, that cholesterol and various bile acids are incorporated in biliary micelles to a various extent. Binding of ouabain and K-strophanthoside to mixed micelles, as well as to non-cholesterol containing micelles occurs; yet an increase of micelle output as the result of taurocholate administration does not lead to an increase of biliary excretion of the 2 cardiac glycosides.

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SUPPLEMENT IV

CHOLERESIS AND HEPATIC TRANSPORT MECHANISMS. IV INFLUENCE OF BILE SALT CHOLERESIS ON THE HEPATIC TRANSPORT OF THE ORGANIC CATIONS, D-TUBOCURARINE AND N^4-ACETYL PROCAINAMIDE ETHOBROMIDE

R.J. VONK, E. SCHOLTENS, G.T.P. KEULEMANS AND D.K.F. MEIJER

SUMMARY

The influence of the bile salts taurocholate and dehydrocholate on the hepatic transport of two quaternary ammonium compounds, d-tubocurarine (dTc) and N^4-acetylprocainamide ethobromide (APAEB) was investigated in rats. The biliary excretion of APAEB and dTc in vivo was not enhanced by 106 µmoles/h of taurocholate or dehydrocholate. Infusion of 268 µmoles/h dehydrocholate caused an inhibition of the plasma disappearance and hepatic transport of dTc. This inhibition, which presumably occurred at the hepatic uptake level, was also observed in isolated perfused rat liver experiments. In animals with an intact renal function, the high dose of dehydrocholate caused a decreased biliary excretion and an increased renal excretion of dTc.

The observed concentration gradients, plasma/liver cytosol and bile/liver cytosol twenty minutes after injection of both drugs were 1.6 and 23 for APAEB and 2.2 and 190 for dTc. These concentration ratios were based on free drug concentrations; corrections were made for plasma protein binding, intracellular binding and biliary micelle binding. No substantial binding of both compounds to ligandin and Z proteins was found. From the amount in the liver,twenty minutes after injection of both drugs 70 per cent of APAEB and 90 per cent of dTc was bound to cellular particles.

The rate limiting step in hepatic transport of APAEB from plasma into bile was concluded to be the hepatic uptake, which may explain the lack of effect of bile salt induced choleresis on its biliary excretion.

INTRODUCTION

The influence of bile salts or bile salt choleresis on hepatic transport of drugs is poorly understood. Administration of bile salts can stimulate biliary excretion of drugs (O'Maille et al., 1966; Ritt and Combes, 1967), while also inhibitory effects of bile salts (Bloomer et al., 1973) were reported. Most of these studies were performed with organic anions, like bromosulphthalein (BSP) (ref. see Vonk et al., 1975).

Less information is available concerning the influence of choleresis on hepatic transport of organic cations. Schanker and Solomon (1963) reported, that bile salts had no influence on the biliary excretion of procainamide ethobromide (PAEB) in the rat, which was confirmed by Kupferberg (1969). However, MacGregor and Clarkson (1972) found a stimulation of biliary excretion of a phenanthridinium salt during dehydrocholate infusion, while recently Kuo and Johnson (1975) reported a stimulatory action of taurocholate on biliary excretion of acetylated PAEB. This discrepancy prompted us to study the characteristics of the hepatic transport of two organic cations, ^{3}H-d-tubocurarine (dTc) and ^{14}C-N^{4}-acetylprocainamide ethobromide (APAEB) and the influence of two bile salts taurocholate and dehydrocholate on this transport. For determination of the characteristics of hepatic transport of both organic cations, the concentration gradients of the free drug between plasma, cytosol and bile were determined. The two compounds are not noticeably metabolized (Cohen et al., 1967; Hwang and Schanker, 1973).

MATERIALS AND METHODS

Chemicals: The chemicals used were obtained from the following sources: d-tubocurarine-chloride: Burroughs Wellcome & Co., London, U.K.; procainamide ethobromide: Squibb & Sons Inc., Princeton, N.J., U.S.A.; sodium dehydrocholate and sodium taurocholate: Fluka A.G., Buchs, Switzerland; mannitol: Merck A.G., Darmstadt, Germany; ^{3}H-d-tubocurarine and ^{14}C-procainamide ethobromide: New Engl. Nuclear Corp., Dreieichenhain, Germany;

^{3}H-taurocholate: New Engl. Nuclear Corp., Boston, Mass., U.S.A.; ^{3}H-d-tubocurarine was further purified according to Meijer et al. (1972). The chemicals used were dissolved in saline before administration to the animals.

Synthesis of APAEB: The synthesis is performed according to the method described by Meijer et al. (1970[a]) with modifications in the amounts of reagents used: 0.25 ml 0.3 M PAEB was mixed with 0.02 ml 10.5 M acetic anhydride, followed by the addition of 0.05 ml 4.2 M sodium acetate. Colorimetric determination indicated that about 97 % of the PAEB was acetylated.

Chemical analysis: APAEB and PAEB were determined colorimetrically according to the method of Bratton and Marshall (1939) with modifications as described by Meijer et al. (1970[a]).

Radiochemical analysis: ^{3}H-dTc, ^{14}C-APAEB and ^{3}H-taurocholate were estimated with a liquid scintillation spectrometer (Nuclear Chicago Mark II). Supernatant, plasma, bile and urine were solubilized in one ml Soluene (Packard Instrument Comp. Inc., Downers Grove, Ill., U.S.A.); liver homogenate and particulate fraction were solubilized in 2.7 ml Soluene by heating six hours at 40^{o}C. Afterwards 10 ml scintillation medium was added to each vial. The scintillation medium consisted of 5 g of 2,5 - diphenyloxazole (PPO) and 50 mg 2,2'-p-phenylen-bis (5-phenyl-oxazol) (POPOP) per liter toluene. The samples were counted at an efficiency of about 85 % for ^{14}C and 35-50 % in case of ^{3}H labeled material. The radioactivity in the eluted fractions after gel filtration were determined by dissolving 0.5 ml of each fraction in 10 ml Aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.). Counting efficiency for ^{3}H and ^{14}C was 37 % and 72 % respectively. Quenching of each sample was corrected for by external standardization.

Experiments in vivo: The experimental procedure in animals has been described previously (Vonk et al., 1975). Blood samples of about 150 µl were taken from the carotid artery and collected in

heparinized capillary tubes (Sherwood, Med. Ind. Inc., St. Louis, U.S.A.). Plasma disappearance studies and biliary excretion studies were performed in separate groups of animals. Biliary and urinary excretion was measured in sampling periods of 15 min. Urine was collected by cannulation of the urine bladder. Ligation of the cannulas was performed in such a way that the dead space in the urine bladder was minimized. For a constant urine production mannitol was dissolved in the infusion liquid and infused in a dose of 1042 µmoles/h. Infusion of a double dose of mannitol increasing urine production twofold did not enhance the urinary excretion of dTc. In all experiments the quaternary ammonium compounds were injected over a period of three minutes to avoid a severe decrease of blood pressure.

Isolated perfused rat liver experiments: The equipment and method used in the perfusion experiments were those reported by Meijer and Weitering (1970[b]) with one modification. In the present experiments the perfusion fluid was composed of Krebs-bicarbonate buffer solution (pH 7,4) with 3 % bovine serum albumin and sheep erythrocytes. By centrifugation, erythrocytes from 80 ml of sheep blood were isolated and washed three times with sterile saline. In this way about 33 ml of erythrocytes were obtained, which were suspended in 100 ml Krebs-bicarbonate-albumin buffer. Glucose and ampicillin were added at concentrations of 2 mg/ml and 25 µg/ml respectively. About 90 ml of the medium were used in the experiments and perfused through the liver via the portal vein and recirculated. The viability of the isolated perfused livers was tested by measuring perfusion flow through the liver, pH of the perfusion medium and the bile flow. Perfusate and bile flow were approximately 20 ml/min and 13 µl/min respectively at the start of the experiment. After an equilibration period of 30 min the bile salts were administered. A priming dose of 55 µmoles was given and a constant infusion of 106 µmoles/h was started (3.8 ml/h). After 30 min dTc was added to the perfusion medium. Bile was collected in 15 min fractions during 90 min; samples of the perfusion medium were taken at t = 10, 20, 30, 40, 50, 60, 90 min, before and after passage

through the liver. Radioactivity of the perfusion medium was determined in the clear supernatant obtained after centrifugation.

Determination of sub-cellular distribution: Twenty min after injection of the drug, the liver was perfused in situ via the portal vein with 50 ml saline and removed from the animal. In contrast to the method described earlier (Meijer et al., 1972) in these experiments a 25 % (w/v) homogenate in 0.01 M sucrose - 0.01 M phosphate buffer (pH 7.4) was prepared, as used for determination of dye binding to cytosol proteins according to Levi et al. (1969). The homogenate was prepared using a Teflon-glass motor driven Potter-Elvehjem homogenizer. The homogenate was centrifugated at 4°C during 120 min at 160.000 g_{av} in a Spinco ultracentrifuge (rotor Ti 50). After centrifugation the supernatant was separated and the sediment was resuspended in sucrose-phosphate buffer and samples of the supernatant and the sediment suspension were taken for determination of drug content. From the supernatant a sample of four ml was subjected to column chromatography using Sephadex G75-S. The column (cross-section 2.5 cm, height 83 cm) was eluted with 0.01 M phosphate buffer pH 7.4 (16 ml/h) at 4°C, and 4.0 ml fractions were collected in which extinction at 280 nm and radioactivity were measured.

Calculation of cytosol concentration: Cytosol concentrations of APAEB and dTc were estimated from the supernatant concentration after ultracentrifugation of liver homogenates, according to the method described by Meijer et al. (1972). Some modifications of that method were introduced: the volume of the biliary tree in rat liver was assumed to be 0.32 % of the liver weight (Forker et al., 1970); 3 volumes of 0.01 M sucrose - 0.01 M phosphate buffer were used instead of 1.5 volume 0.15 M KCl; redistribution between cell organelles and cytosol during homogenisation was assumed not to occur. Drug concentration in the hepatic extracellular space was neglected, because vascular perfusion of the liver in situ with 50 ml saline will remove most of the extracellular drug, assuming that the exchange of drug between plasma and extracellular space outside plasma is sufficiently rapid.

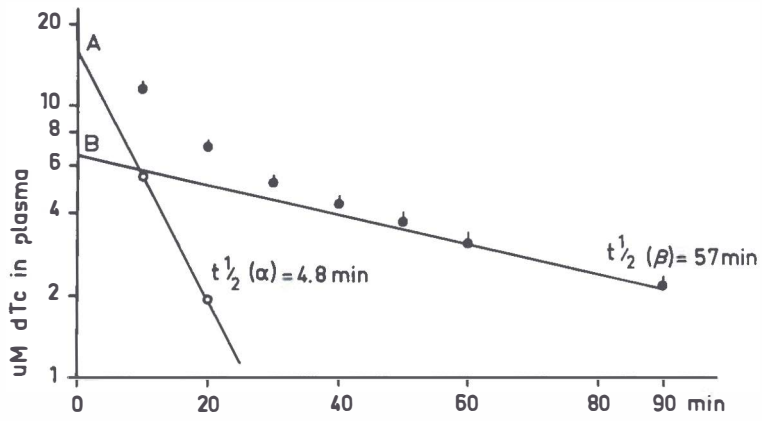

Fig. 1. Plasma disappearance curve of dTc (4.2 µmoles/kg b.wt.) in rats with ligated renal pedicles during infusion of saline. Mean value ± S.E. (n = 6).

Table I

	saline	TC 106 µmoles/h	DHC 106 µmoles/h	DHC 268 µmoles/h
plasma disappearance:				
$t_{\frac{1}{2}}$ (α) (min)	4.8 ± 0.7	6.6 ± 0.8	6.7 ± 0.6	6.2 ± 0.7
$t_{\frac{1}{2}}$ (β) (min)	57 ± 7	67 ± 12	109 ± 20*	133 ± 10*
biliary excretion: (descending phase)				
$t_{\frac{1}{2}}$ (min)	34 ± 1	38 ± 2	38 ± 1	31 ± 1

Table I. Pharmacokinetic parameters of dTc in rats with ligated renal pedicles. Injection of 4.2 µmoles/kg b.wt. (n = 6). Mean value ± S.E. * Significantly deviating from controls.

Cytosolic concentration was calculated in this case from the expression: $C_{cyt} = 8.098\ C_{sup} - 0.007\ C_{bile}$. (Derived from calculations of Meijer et al., 1972).

Binding of drugs to biliary micelles: Binding of the drugs to biliary micelles was studied by ultracentrifugation of bile, according to the procedure described by Vonk et al. (1977). The drugs were added to bile in vitro to a concentration of 0.38 mM and 0.88 mM for dTc and APAEB respectively. In experiments in which the sedimentation of ^{3}H-taurocholate in bile was measured, a tracer amount of ^{3}H-taurocholate was administered to rats via the tail vein, after which bile was collected.

Dialysis experiments of plasma and bile: Dialysis was performed according to Meijer et al. (1976). In the present experiments one ml of plasma and bile was used and dialysed against two ml Krebs-bicarbonate.

Statistical analysis: Statistical comparisons were made using Wilcoxon's test. Unless specified otherwise, the term significant means a P value of less than 0.05.

RESULTS

A. *Effects of bile salts on plasma disappearance, biliary and urinary excretion of dTc*

The plasma disappearance of dTc after injection of 4.2 µmoles/kg in rats with ligated renal pedicles is shown in fig. 1. The plasma disappearance curve seemed to be at least biphasic and exhibited a slow component (β) with a $t_{\frac{1}{2}}$ of 57 ± 7 min and a fast component (α), which has a $t_{\frac{1}{2}}$, corrected for the slower component of 4.8 ± 0.7 min. The distribution volume (v_d) could be calculated from the equation $V_d = D/(A + B)$. D represented the dose and A and B the intercepts of the Y axis with the fast and slow phase respectively. V_d was 58 ml (19 % of b.wt.), which suggests that the distribution of dTc was not

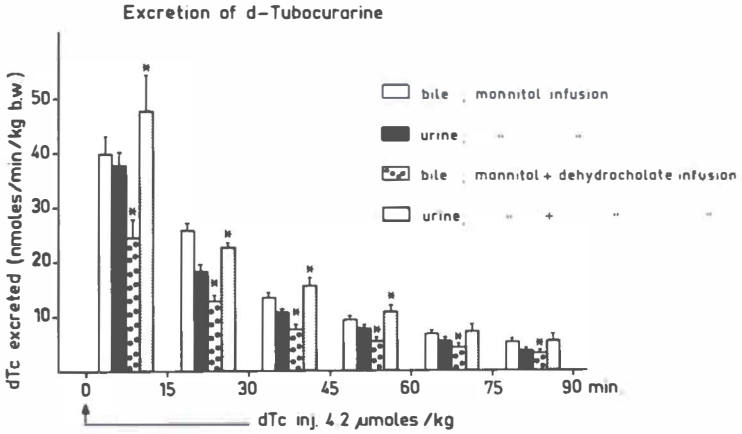

Fig. 2. Biliary and urinary excretion of dTc (4.2 µmoles/kg b.wt.) during infusion of a. saline and mannitol (1042 µmoles/h)(n = 6), b. dehydrocholate (268 µmoles/h) and mannitol (1042 µmoles/h) (n = 6). Mean values ± S.E. ✱ Significantly deviating from controls.

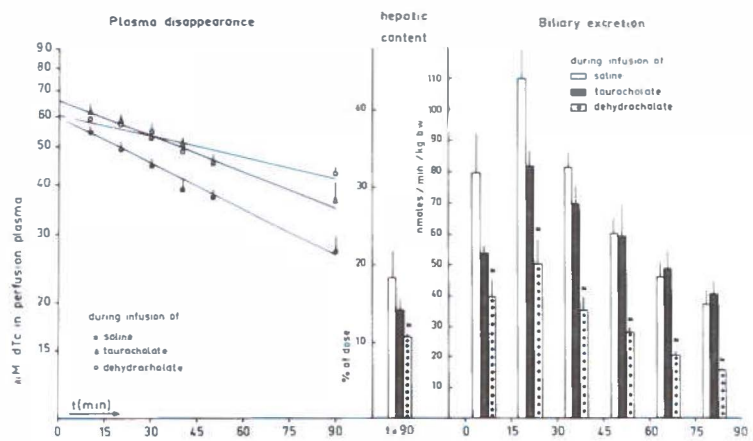

Fig. 3. Plasma disappearance, liver content at t = 90 min and biliary excretion of dTc in isolated perfused rat liver experiments after addition of 5.2 µmoles dTc during infusion of saline (n = 6), taurocholate (106 µmoles/h) (n = 3) and dehydrocholate (106 µmoles/h) (n = 3). Mean values ± S.E. ✱ Significantly deviating from controls.

restricted to the plasma volume. Administration of bile salts prolonged both $t_{\frac{1}{2}}$'s (Table I). This effect was most pronounced during administration of dehydrocholate: $t_{\frac{1}{2}}$ (β) was increased from 57 min to 109 min (106 µmoles/h) and 133 min (268 µmoles/h).

Biliary excretion of dTc was measured in rats with ligated renal pedicles. The $t_{\frac{1}{2}}$ of the descending phase of the biliary excretion curve was 34 $\pm$ 1 min (Table I). An infusion of 106 µmoles/h of taurocholate or dehydrocholate caused no significant changes in biliary excretion of dTc. Administration of 268 µmoles/h dehydrocholate, however, inhibited biliary output of dTc significantly in all periods. The total biliary excretion in this case was 32 % of the dose compared with 55 % in control experiments. However, the $t_{\frac{1}{2}}$'s of the descending phase of the biliary excretion curves were not significantly changed by administration of bile salts (Table I).

Biliary and urinary excretion of dTc during infusion of mannitol and saline were measured in rats with an intact renal function (Fig. 2). The cumulative urinary excretion in 90 min was slightly smaller than the total biliary excretion (30 % and 36 % respectively), while biliary excretion in this case was smaller than in experiments with ligated renal pedicles (36 % and 55 % respectively). Compared with controls with intact renal pedicles, administration of the high doses of dehydrocholate again decreased biliary excretion of dTc from 36 % to 22 %, but the urinary excretion was enhanced from 30 to 44 %.

To minimize interactions of bile salts with dTc outside the liver and to exclude extrahepatic toxic effects of high doses of dTc, the technique of the isolated perfused liver was used to study the pharmacokinetics of a higher dose of dTc (5.2 µmoles) and the influence of bile salts on its pharmacokinetics. The plasma disappearance in the perfused liver experiments, which was linear when plotted semilogarithmically, could be described by a one compartment model (Fig. 3). The pharmacokinetic values V_d, $t_{\frac{1}{2}}$ and K_{cel} (clearance constant) were found to be 93 $\pm$ 8 ml, 82 $\pm$ 7 min and 0.8 $\pm$ 0.1 ml.min^{-1} respectively. The same phenomena as observed in vivo were found: the plasma disappearance of dTc was retarded by dehydrocholate. In the isolated perfused liver

dehydrocholate appeared to be more effective than in intact animal studies. In this case the inhibitory effects could be observed with 106 µmoles/h. The amount of dTc in the liver was significantly diminished after dehydrocholate administration. Also the biliary output was reduced by bile salt administration. The total amount excreted in bile was significantly reduced by the hydrocholate (16 per cent vs. 35 per cent in control experiments). The $t_{\frac{1}{2}}$ of the biliary excretion process was 44 ± 8 min, which was not significantly changed by dehydrocholate and only slightly reduced by taurocholate.

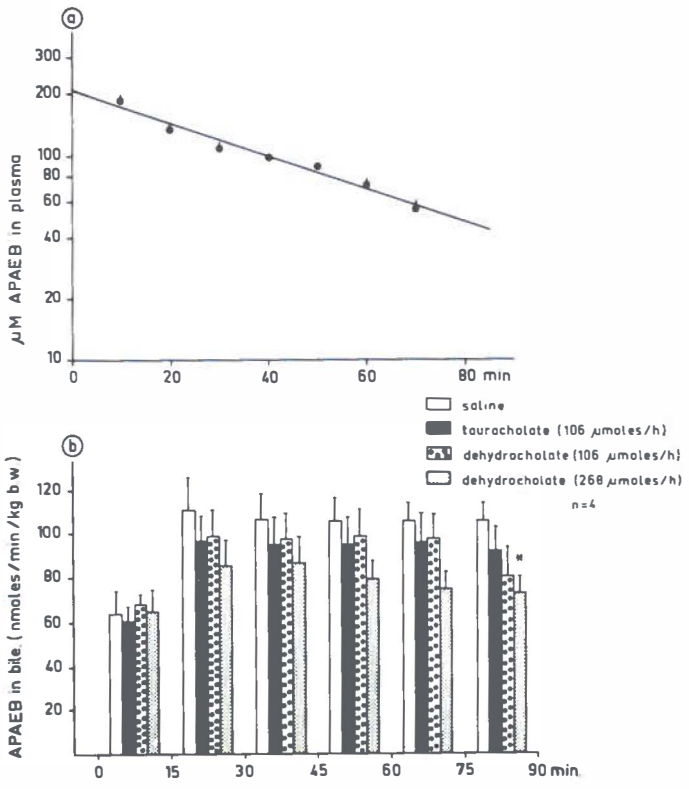

Fig. 4. Pharmacokinetics of APAEB in rats with ligated renal pedicles after i.v. injection of 35.3 µmoles/kg b.wt. of APAEB.
a. Plasma disappearance of APAEB. Mean values ± S.E. (n = 4)
b. Biliary excretion of APAEB during infusion of saline, taurocholate (106 µmoles/h) and dehydrocholate (106 and 268 µmoles/h). Mean values ± S.E. * Significantly deviating from controls.

B. Effect of choleresis on hepatic transport of APAEB

Plasma disappearance of APAEB in vivo after injection of 35.3 µmoles/kg is shown in fig. 4a. It is not clear, whether the plasma disappearance of APAEB should be described by a one compartment model or a more complex model. For an approximation of the pharmacokinetic parameters, we have assumed a one compartment model and after calculating the best fitting line, V_d and $t_{\frac{1}{2}}$ were found to be 52 ml and 38 min respectively. The biliary excretion of APAEB is shown in fig. 4b. After injection of 35.3 µmoles/kg of APAEB a maximal hepatic transport rate of 111 nmoles/min/kg b.wt. was found, which could not be enhanced by administration of bile salts. A decrease of the hepatic transport rate, which is only significant in the last period, was found during administration of dehydrocholate (268 µmoles/h). In fig. 5 biliary excretion of APAEB and PAEB is shown after injection of an equimolar dose of 35.5 µmoles/kg of PAEB. (PAEB is metabolized in the liver to APAEB; Meijer et al., 1970[a]). After injection of PAEB maximal biliary excretion of APAEB amounted to 101 nmoles/min/kg b.wt., in addition to a maximal biliary excretion of PAEB of 211 nmoles/min/kg b.wt. In this situation biliary excretion of APAEB was only 9 % less than after administration of 35.3 µmoles/kg b.wt. of APAEB.

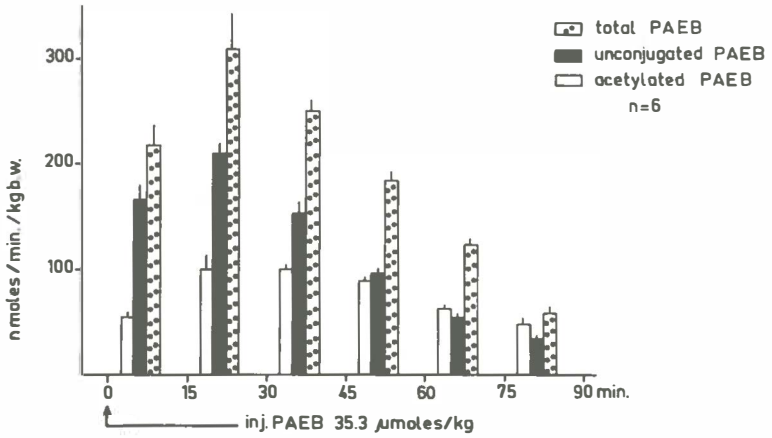

Fig. 5. Biliary excretion of PAEB and APAEB during 90 min after i.v. injection of 35.3 µmoles/kg b.wt. of PAEB in rats with ligated renal pedicles.

Table II

	dTc 4.2 µmoles/kg	APAEB 35.3 µmoles/kg
% liver	12.0 ± 0.4	9.2 ± 0.3
% bile	25.9 ± 1.9	4.3 ± 0.3
% distribution volume (V_d)	43.1 ± 2.7	61.4 ± 1.6
% total rec.	81.0 ± 8.6	74.9 ± 2.2
conc. bile (µM)	860 ± 56	1584 ± 135
conc. bile (free) (µM)	550	1378
conc. plasma (µM)	9.4 ± 0.6	114 ± 3
conc. plasma (free) (µM)	6.3	98
conc. cytosol (µM)	2.9 ± 0.3	60.0 ± 4.9
conc. cytosol (free) (µM)	2.9	60.0
partition ratio cytosol/particles	0.11	0.43
plasma/cytosol ratio	2.2	1.6
bile/cytosol ratio	190	23

Table II. Distribution of dTc and APAEB *in vivo* twenty minutes after injection of the compound. The percentages in liver, bile and distribution volume and total recovery are percentages of the injected dose. Mean value ± S.E.; n = 4.

C. Concentration ratio plasma/cytosol and bile/cytosol of unbound dTc and APAEB

To characterize hepatic transport of both organic cations in order to enable interpretation of the influence of choleresis on this transport, concentration gradients between plasma, cytosol and bile were determined. An injection of dTc (4.2 µmoles/kg) and APAEB (35.3 µmoles/kg) was given to two groups of animals with ligated renal pedicles and twenty min afterwards the concentration of both drugs was determined in plasma, bile liver

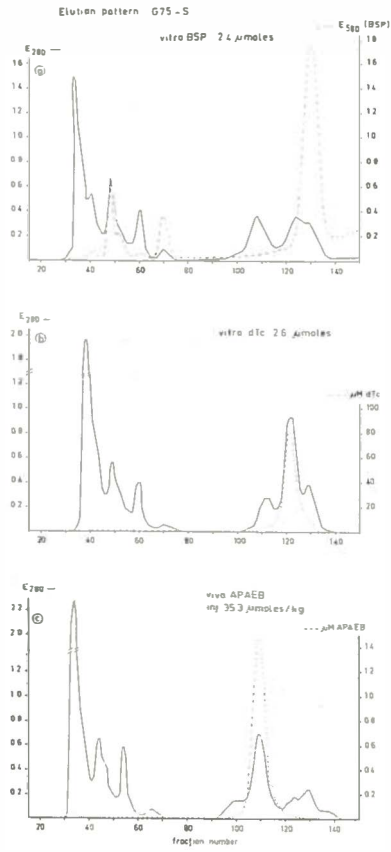

Fig. 6. Elution patterns of cytosol from G 75-S Sephadex columns to which was applicated:

(a) cytosol with addition of 2.4 µmoles BSP

(b) cytosol with addition of 2.6 µmoles dTc

(c) cytosol, obtained from rats, which were injected with APAEB (35.3 µmoles/kg)

homogenate and in post microsomal supernatant and sediment of liver homogenate after centrifugation. Cytosol concentrations were calculated as described in "methods" and the results are given in table II. The recovery of dTc and APAEB in liver, bile and plasma was 81 and 75 % of the dose respectively. Both compounds were found to be bound to cell organelles after injection in vivo and homogenisation of the liver.

Concentration ratios should be based on free drug concentration in plasma, cytosol and bile. Therefore, the concentration measured in plasma, cytosol and bile was corrected for binding to plasma proteins, cytosol proteins and biliary micelles respectively.

Dialysis studies revealed that 46 % of dTc (Meijer et al., 1976) and 14 % of APAEB was bound to plasma proteins in the concentration range used in the in vivo experiments.

To investigate binding of both drugs to cytosol proteins, supernatant after centrifugation of liver homogenate was applied to a Sephadex G 75-S column. Both the supernatant prepared from livers of animals which were injected with dTc or APAEB *in vivo*, and the supernatant obtained from the liver of control rats to which both drugs were added *in vitro* were investigated. Fig. 6 shows the elution pattern when a. 2.4 µmoles BSP were added in vitro to liver supernatant (reference) b. 2.6 µmoles dTc were added in vitro and c. an injection of 35.3 µmoles/kg APAEB was given in vivo and liver supernatant was prepared. The elution pattern of the postmicrosomal supernatant after addition of BSP shows the well-known pattern as earlier reported by Levi et al. (1969). dTc and APAEB were almost completely eluted in the peak of low molecular weight compounds (V_t).

Interaction of APAEB and dTc with biliary micelles was studied by ultracentrifugation and dialysis of bile containing both drugs. Fig. 7 represents the sedimentation patterns after centrifugation of respectively bile, to which dTc (0.38 mM) was added in vitro, bile to which APAEB (0.88 mM) was added in vitro and bile which contained tracer amounts of ^{3}H-taurocholate. The sedimentation pattern of ^{3}H-taurocholate is similar to the one reported for taurocholate (Vonk et al., 1977). The higher

concentration of dTc and APAEB in the bottom fraction after sedimentation indicates binding to biliary micelles of both compounds. Also when dTc and APAEB were injected to rats and bile was collected and centrifugated by the method described by Vonk et al. (1975), sedimentation occurred. The amount of drug, which was bound to biliary micelles was estimated according to the method described by Vonk et al. (1977). After 17 h of centrifugation 25 % of APAEB and 59 % of dTc was bound to mixed biliary micelles. Dialysis experiments of bile containing dTc and APAEB revealed different binding percentages, which were 13 % and 36 % respectively.

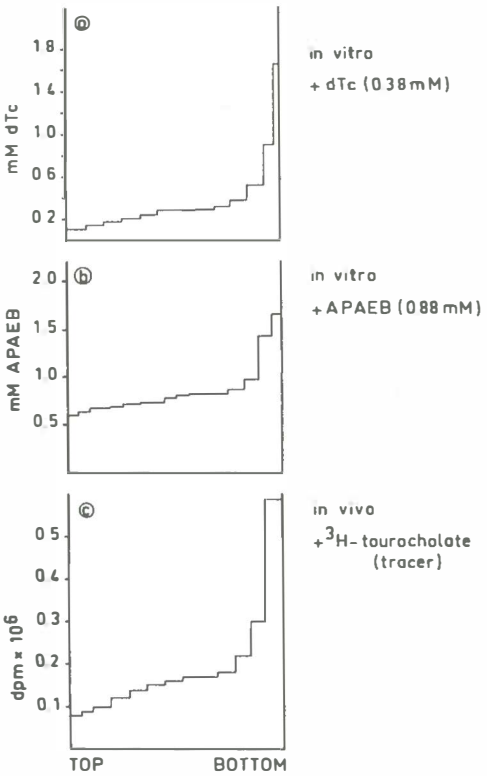

Fig. 7. Sedimentation patterns after centrifugation of:
(a) bile to which dTc was added in vitro (0.38 mM)
(b) bile to which APAEB was added in vitro (0.88 mM)
(c) bile collected after injection of a tracer amount ^{3}H-taurocholate

When corrections were made for binding to plasma proteins, cytosol proteins and biliary micelles it was found (Table II) that the free concentration of both drugs in plasma was higher than in cytosol, and a concentrative step existed in the transfer from cytosol to bile.

DISCUSSION

In several studies the pharmacokinetics of quaternary ammonium compounds have been investigated (refs. see Cohen et al., 1965; Meijer, 1977). In spite of this, the pharmacokinetics of dTc and APAEB are not completely understood due to uncertainties in analysis of the plasma disappearance and biliary excretion curves, which is for example illustrated in the plasma disappearance of APAEB (Fig. 5a). The plasma disappearance of dTc in rats with ligated renal pedicles is composed of at least two components, but the second component is not only determined by biliary elimination if we take into account the $t_{\frac{1}{2}}$ of plasma disappearance and biliary excretion (Table I). The same phenomenon could also be observed in isolated perfused liver experiments: the $t_{\frac{1}{2}}$ of biliary excretion was considerably smaller than the $t_{\frac{1}{2}}$ of plasma disappearance.

When experiments with dTc were performed in animals with intact renal function, considerable amounts of dTc were excreted in urine, while the cumulative biliary excretion in these animals was lower.

The liver takes up both quaternary ammonium compounds while the free drug concentration ratio plasma/cytosol > 1. (Table II). This can be partly explained by a high binding to cell organelles, for dTc especially to lysosomes (Weitering et al., 1975, 1977). From the amount in the liver twenty minutes after injection of both drugs 70 per cent of APAEB and 90 per cent of dTc was bound to cellular particles. (The partition ratio liver cytosol/ cellular particles was 0.11 for dTc and 0.43 for APAEB; Table II). Binding to the cytosol proteins Y and Z was not substantial; APAEB, dTc and PAEB were not eluted in one of these protein fractions after columnchromatography. This is in contrast to the

organic anions, for instance BSP, which was eluted in the Y and Z fractions. Ligandin and Z protein are proteins which play a role in the hepatic transport by lowering the free cytoplasmic concentrations of organic anions like BSP. Both quaternary ammonium compounds are partly bound to plasma proteins and biliary micelles. The latter was determined by ultracentrifugation and dialysis of bile. One of the reasons causing the differences in percentage of binding determined by both methods may be the dilution in the dialysis technique, which may diminish the amount of biliary micelles and/or promote dissociation from the micelles and as a consequence the percentage of binding. The values derived from the ultracentrifugation method were used for calculating the free canalicular drug concentration.

In our experiments administration of taurocholate or dehydrocholate, which enhanced bile flow, did not stimulate hepatic transport of the quaternary ammonium compounds. In vivo biliary output of dTc as well as APAEB was not enhanced during administration of 106 µmoles/h of bile salts. Also in isolated perfused liver experiments, where the number of interactions outside the liver is reduced, no stimulation of biliary output of dTc could be observed. The same applied to PAEB; no stimulation of the biliary excretion was found by bile salt administration (unpublished observation). Our findings confirm the results of Schanker and Solomon (1963) and Kupferberg (1969), who reported that the excretion of quaternary ammonium compounds can not be stimulated by administration of bile salts. However, two other papers reported a stimulatory effect of bile salts on hepatic transport of quaternary ammonium compounds. MacGregor and Clarkson (1972) found a distinct increase of biliary excretion of a phenanthridinium salt during dehydrocholate infusion. This phenanthridinium salt, which is metabolized by the liver, formed complexes with bile. They suggested that interaction of organic cations with biliary micelles may facilitate biliary excretion of the compound. This cannot be a general phenomenon, because we also observed interactions between dTc and APAEB and biliary micelles without finding a stimulatory effect of taurocholate, which easily forms biliary micelles. Also with N-methyldeptropine,

a quaternary ammonium compound which seemed to form complexes with biliary compounds (Lavy et al., 1972), no stimulation of biliary excretion could be observed by bile salt administration (unpublished observation). The stimulatory effect of a bolus injection of taurocholate on biliary excretion of APAEB, described by Kuo and Johnson (1975) could not be reproduced in our animals, using their experimental set up. The discrepancy of the results from above mentioned groups and our findings is difficult to explain. Drugs, used to induce choleresis, may interact at different sites with the transport system of drugs. The influence of choleresis therefore may be the result of both inhibitory and possible stimulatory effects. The final result may depend on the dose and the type of the choleretic agent administered, the way of administration of the drug to be studied and its physicochemical properties. An example of this complexity are the results with dehydrocholate, which is able, under certain circumstances, to inhibit hepatic transport of drugs, in spite of a distinct choleresis: ouabain (Kupferberg, 1969), phalloidin (Lutz et al., 1971), bilirubin (Bloomer et al., 1973) rose bengal (Kelman-Sraer et al., 1973), indocyanine green (Vonk et al., 1974) bromsulphthalein (Delage et al., 1975) and K-strophanthoside (Vonk et al., 1977). Because of the variety in structure of these compounds general competitive interactions are less likely. Whether this is a specific effect of dehydrocholate and/or metabolytes or a more general bile salt effect is not clear from these experiments. Recently it was observed in isolated hepatocyte suspensions that both dehydrocholate and taurocholate were able to inhibit cellular transport of various compounds, which suggests a more general effect (Vonk et al., 1978). Whether there is any correspondence with the action of K-strophanthoside on hepatic transport of dTc (Meijer et al., 1972), has yet to be established.

The studies with isolated hepatocytes furthermore indicated, that inhibition occurred at the uptake level, which may be confirmed in these studies. In vivo $t_{\frac{1}{2}}$ of the biliary excretion was not affected by bile salts, while the total biliary output was reduced and in isolated perfused liver experiments both the

amount excreted in bile and the amount in the liver were reduced.

The inhibition of hepatic uptake in vivo has also consequences for the urinary excretion of drugs, as seen with dTc. During administration of dehydrocholate which inhibits hepatic uptake, plasma levels of dTc were increased, which, in turn, increased renal excretion of dTc.

Inhibition of hepatic uptake of drugs by bile salts may also occur in pathological situations as cholestasis, in which serum bile salt levels may be high (La Russo et al., 1975). Whether these high serum bile salt levels also can prolong the duration of action of neuromuscular blocking agents like dTc is unknown. The observation, that in patients with a total biliary obstruction, the neuromuscular blockade of the organic cation pancuronium was prolonged (Somogyi et al., 1977), seems to suggest this possibility.

Administration of taurocholate or dehydrocholate did not stimulate biliary excretion of the quaternary ammonium compounds dTc and APAEB. This is in contrast with the stimulation of the hepatic transport of the organic anions indocyanine green or dibromosulphthalein, which was studied under the same experimental conditions (Vonk et al., 1975). Therefore the mechanism and rate limiting step of the hepatic transport of both quaternary ammonium compounds were analyzed. The transport of dTc can be described by a non concentrative step from plasma to cytosol and a concentrative step from cytosol to bile. The same conclusion was drawn from data obtained under different experimental conditions (Meijer et al., 1972). The rate limiting step in the hepatic transport of dTc is not quite clear. From the observation of Meijer et al. (1976) that the free dTc concentration in cytosol is decreasing during 90 min after an initial rise, it is suggested that the rate limiting step is the biliary excretion process.

If the stimulation of biliary excretion of drugs in general is due to a modification of the canalicular membrane and/or transport carrier to bile (Forker, 1973), transport of dTc is not affected by these modifications. If the stimulation of biliary excretion of drugs is a flow dependent process

including back transport from bile to liver (Vonk et al., 1976), the lack of effect of choleretics can be explained by the assumption that transport from bile to liver does not exist in case of dTc.

The hepatic transport of APAEB can also be described by a non concentrative step from plasma to cytosol and a concentrative step from cytosol to bile. The rate limiting step turned out to be the hepatic uptake. This conclusion could be derived from the comparison of biliary excretion of APAEB after injection of APAEB and PAEB. After injection of PAEB a biliary excretion of 211 nmoles/min/kg b.wt. of PAEB was found, while the simultaneous excretion of its acetylated conjugate (APAEB) was only 9 % less than after injection of APAEB itself. This implies that the uptake of PAEB is much faster than hepatic uptake of APAEB and that hepatic uptake rather than biliary excretion is the rate limiting step in hepatic transport of APAEB. This is based on the assumption that APAEB and PAEB are transported by the same carriers to bile. If the site of the stimulatory action of bile salts is distal to the rate limiting uptake process, this stimulatory effect should not be visible in experiments with APAEB. This might be the explanation for the lack of effect of bile salt choleresis on hepatic transport of APAEB.

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SUPPLEMENT V

TRANSPORT OF DRUGS IN ISOLATED HEPATOCYTES THE INFLUENCE OF BILE SALTS

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Abstract—The influence of bile salts on hepatic transport of drugs was studied using isolated hepatocyte suspensions. *Uptake* of the organic anions, dibromosulphthalein (DBSP), indocyanine green (ICG) and an organic cation, N^4-acetyl procainamide ethobromide (APAEB) was measured. After 60 min incubation the amount of DBSP, ICG and APAEB present in the cells was 17, 41 and 4.5 per cent of the added amount respectively. The *release* of DBSP, ICG and APAEB from the hepatocytes preincubated with the agents under study, after 60 min incubation in fresh medium was 80.5, 12.5 and 48.9 per cent of the amount initially present respectively. The presence of bile canalicular membranes in the isolated hepatocytes was demonstrated by enzymehistochemistry: 5'-nucleotidase activity showed sharp branched bands over the cell surface. When bile salts were present in the incubation medium, the cellular content of DBSP, ICG and APAEB was diminished. The taurocholate concentration which caused 50 per cent of the maximal effect was 0.07 mM, 0.10 mM and 0.06 mM in experiments with DBSP, ICG and APAEB respectively. Pharmacokinetic analysis revealed that the influence of bile salts on cellular content of the three compounds was due to inhibition of the uptake into the isolated hepatocytes, rather than stimulation of release from the cells. The hypothesis, that stimulation of biliary output of organic anions *in vivo* is due to a modifying effect of bile salts on the canalicular membranes, instead of being the result of the increased bile flow, is not supported by this study.

Bile salts are able to affect the hepatic transport of drugs *in vivo*. In some studies a stimulation of biliary excretion of drugs is found [1–4], while other studies revealed inhibition of hepatic transport of drugs* [5] or lack of effect* [6]. No definite explanation for the observed differences in effects of bile salts can be given at this moment. The mechanism of the inhibitory effect of bile salts on hepatic transport is not clear. The stimulatory effect of bile salts may be due to a direct effect of the bile salts on the carrier for biliary transport of drugs or to an indirect effect of the bile salts. The direct effect of bile salts may be the "allosteric" effect described by Forker [7] or a detergent effect of bile salts on plasma membranes [8, 9]. The indirect effect is the effect of the concomitant choleresis induced by bile salts, which is supposed to change the canalicular concentration of the drug and thereby the net transport from liver to bile [1]. Previous studies [10] revealed that the stimulatory influence of bile salts on hepatic transport depends on the drug under study and both the membrane modifying effect of bile salts and the effect due to choleresis, may play a role. Also the existence of a concentration gradient of drugs along the sinusoids from the portal triad to central vein [11] may complicate the interpretation of the interaction between drugs and choleretic agents.

The present study is concerned with the influence of bile salts on hepatic transport of drugs using the technique of isolated hepatocytes. The advantage of this technique is the exclusion of the factor of dilution of canalicular drug concentration by choleresis and the lack of an unequal supply of drug to hepatocytes from the various parts of the liver lobule. Possible direct effects of bile salts on membranes, which will influence transport of drugs are subject of this investigation.

Since the normal orientation of hepatocytes in the liver lobule is lost during the isolation procedure, enzymehistochemical studies were performed to investigate whether bile canalicular membranes, mediating canalicular transport of drugs, were still present in the isolated hepatocytes.

MATERIALS AND METHODS

Chemicals. The chemicals used were obtained from the following sources: DBSP: Société d'Etudes et de Recherches Biologique (SERB), Paris, France; ICG: Hynson, Westcott and Dunning Inc., Baltimore, U.S.A.; PAEB: Is a gift from Squib and Sons, New Jersey, U.S.A.; APAEB: by acetylation of PAEB, according to the procedure described by Vonk *et al.*;* Sodium taurocholate and sodium dehydrocholate: Fluka, A. G., Buchs, Switzerland; [^{14}C]PAEB: New Engl. Nuclear Corp., Dreieichenhain, Germany; [^{3}H]taurocholic acid: New Engl. Nuclear Inc., Boston, U.S.A.; Albumin: demineralized bovine albumin, Poviet, Oss, Holland; Collagenase: (EC 3.4.24.3) Type I, Sigma Chemical Company, St. Louis, MO 63178, U.S.A.

Chemical analysis. DBSP: supernatant (medium) was diluted with 0.1 N NaOH and measured spectrophotometrically at 580 n m; hepatocytes were extracted with 80% methanol–20% 0.1 N NaOH and after centrifugation (Homef, 700 *g*) the supernatant

* Vonk *et al.*, to be published.

was analyzed spectrophotometrically at 580 nm. Recovery in medium and hepatocytes was about 100 per cent.

ICG: supernatant was diluted with distilled water and measured spectrophotometrically at 800 nm. The pellet of hepatocytes was extracted with 100% methanol and after centrifugation (Homef, 700 *g*) the supernatant was analyzed spectrophotometrically at 800 nm. Recovery in medium and hepatocytes was about 100 per cent.

[^{14}C]APAEB: 100 μl of supernatant was dissolved in 10 ml Aquasol (New England Nuclear Corp., Boston, MA, U.S.A.) and the radioactivity was estimated with a liquid scintillation spectrometer (Nuclear Chicago Mark II). The pellet, consisting of cell material was suspended in 3 ml Krebs-buffer; 1 ml was dissolved in 10 ml Aquasol. Quenching of each sample was corrected by external standardization.

Isolation of hepatocytes. Hepatocytes were isolated according to the procedure of Berry and Friend [12] with some modifications.

Male Wistar rats (280–330 g), which had free access to food and water, were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal®) (60 mg/kg). After a midline abdominal incision the liver was exposed and the vena porta cannulated. Immediately afterwards the lower part of the vena cava inferior was intersected and the liver perfused with a Ca^{2+}-free Hanks buffer. The colour of the liver instantaneously became uniformly yellow. During preperfusion the upper part of the vena cava inferior was cannulated and the liver transported to a perfusion apparatus [13], which was constructed for these experiments of plexiglass. After 8 min of preperfusion with a flow of about 35 ml/min, a recycling perfusion with 60 ml Hanks buffer, containing 0.03% collagenase (200 U/mg) was started. During the perfusion as well as during the preperfusion the solutions were constantly gassed with carbogen, the temperature was maintained at 37° and the pH constantly monitored (pH 7.17.4). The perfusion was stopped after 20 min and the liver was transferred to a petridisk and gently disrupted with two pincets. The formed suspension was incubated in a Dubnoff metabolic shaker (37°, 5 min, aerated in open air, ±90 rev/min). Afterwards the suspension was filtered through two nylonlayers with cross section of 100 and 60 μm. The filtrate, cooled on ice was centrifugated in a Homef centrifuge (90 sec, 50 *g*). The supernatant was discarded and the pellet resuspended in an ice-cooled buffer, pH 7.4, containing 121.0 mM NaCl, 4.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.13 mM $CaCl_2$, 16.5 mM Na_2HPO_4, 5 mM glucose and 2% bovine serum albumin. After washing twice, the cells were suspended in a known volume of the same buffer. Samples of this suspension were used for the determination of the number of cells and dry weight. The cells were stored on ice during about 1 hr after isolation. Cell suspension with a concentration of 5.0×10^6 cells/ml were used in all experiments.

Viability tests. Trypan blue staining showed that more than 98 per cent of the cells did not take up the dye. Potassium content, electronmicroscopy and enzymehistochemistry of hepatocytes taken after the isolation and incubation procedures did not reveal distinct cellular deviations from hepatocytes of intact liver. Intracellular potassium concentrations were measured by flame photometry. After centrifugation (90 sec, 50 *g*) of a known volume of cell suspension, the pellet was thoroughly mixed with 2 ml 15% trichloroacetic acid. After another centrifugation, one ml of the supernatant was added to 0.5 ml 65% HNO_3 and heated overnight at 80–100°. The residue was dissolved in distilled water and used for flame photometry. The volume of 127×10^{-6} cells was assumed to be 1.0 ml [14]. The intracellular potassium concentration determined in this way was in the range of 104–140 mM.

Uptake studies. The cell suspension, divided in portions of 3 ml was preincubated 10 min in the Dubnoff metabolic shaker (37°, aerated in open air). Afterwards the drug under study was added and incubated under the same circumstances during various time intervals (5, 10, 15, 20, 25, 30, 45, 60 and in some experiments 120 min). The uptake process was stopped by placing the tubes on ice; no changes in drug concentration in cells or in medium were observed during incubation on ice. To separate the supernatant, the suspension was centrifuged (Homef, 90 sec, 50 *g*). After washing the cells twice with the before mentioned buffer, the drug content in the cells was determined. Also in the first supernatant (incubation medium) the amount of drug was analyzed. In experiments, in which the influence of bile was measured 5 min preincubation time with bile salts was used.

Release studies. The cell suspension was preincubated 10 min in the Dubnoff metabolic shaker (37°, aerated open air). The compounds were added and during 30 min incubation was performed under the same conditions. The cells were washed twice with the ice-cooled buffer, resuspended in the same solution and incubated again in portions of 3 ml at various time intervals (5, 10, 15, 20, 25, 30, 45 and 60 min); the incubation was stopped by placing the tube on ice and the amount of the drug was determined in cells and in incubation medium. Control studies showed that no release of drug occurred at 0°.

Enzymehistochemical studies of three plasmamembrane bound enzymes. Cell suspension was allowed to sediment on glass slides. After fixation in formaline–macrodex–calciumchloride (10 ml 40% formaldehyde, 90 ml 6% macrodex in 0.9% sodiumchloride, 1 g calciumchloride), the activities of 5'-nucleotidase and ATP-ase were determined [15]. After fixation at 4° for 3 min in equal parts of chloroform and acetone the activity of leucyl-β-naphthylamidase was visualized [16]. To determine the influence of collagenase on these enzyme activities, cryostat sections (10 μm) of intact liver were preincubated with a 0.05% solution of collagenase in Tris–HCl buffer (0.005 M; pH 7.2) during 30 min.

Pharmacokinetic analysis. Pharmacokinetic analyses were made according to a closed two compartment model, which includes a cellular compartment (1) and a medium compartment (2). Both rate constants r_{12} and r_{21} represent cellular uptake and cellular release respectively. The rate constants can be calculated from *release* studies according to the following equations:

(D = dose and Q = amount ot drug)

$$\frac{dQ_1(t)}{dt} = -r_{12}Q_1(t) + r_{21}Q_2(t); \qquad (1)$$

$$\frac{dQ_2(t)}{dt} = r_{12}Q_1(t) - r_{21}Q_2(t); \quad (2)$$

$$Q_1(t) = \frac{D}{r_{12} + r_{21}}[r_{21} + r_{12}e^{-(r_{12}+r_{21})t}]; \quad (3)$$

it follows that

$$Q_1(\infty) = \frac{D r_{21}}{r_{12} + r_{21}}; \quad (4)$$

$$Q_2(t) = \frac{D r_{12}}{r_{12} + r_{21}}[1 - e^{-(r_{12}+r_{21})t}]; \quad (5)$$

it follows that

$$Q_2(\infty) = \frac{D r_{12}}{r_{12} + r_{21}}; \quad (6)$$

$$\ln[Q_1(t) - Q_1(\infty)] = -(r_{12} + r_{21})t + \text{constant}. \quad (7)$$

With this equation $(r_{12} + r_{21})$ can be calculated graphically by plotting $\ln[Q_1(t) - Q_1(\infty)]$ versus time. After equilibrium in cells and medium $Q_1(\infty)$ and $Q_2(\infty)$ were determined. $Q_1(\infty)/Q_2(\infty) = r_{21}/r_{12}$; now r_{12} and r_{21} can easily be calculated using equations (4), (6) and (7). In *uptake* studies similar calculations can be made.

RESULTS

Uptake studies. Isolated hepatocytes were used to study the uptake of DBSP, ICG and APAEB. Fig. 1 shows that the three compounds were taken up into the cells at different rates: the uptake of DBSP, being 62 nmoles in 60 min, represented 17 per cent of the dose. For ICG and APAEB these percentages amounted 41 and 7.5 respectively. The cellular accumulation of DBSP reaches an equilibrium after about 30 min, in which state the amounts of the drug taken up and released by the cells are equal. Uptake of ICG and APAEB is linear for a longer period. Because an equilibrium was reached with DBSP a pharmacokinetic analysis of the uptake experiments according to a closed two compartment model with this drug could be performed. In this analysis the cells were regarded as compartment one and medium as compartment two, allowing the calculation of rate constants for both release and uptake processes, being 0.094 min^{-1} (r_{12}) and 0.020 min^{-1} (r_{21}) respectively.

The uptake at 0° was very small, which indicates that the presence of the material determined in the cell fraction is not due to unspecific absorption at the outside of the cell membrane. Uptake at 37° occurred in spite of the presence of 2% bovine serum albumin in the medium, which binds both DBSP and ICG with high affinity.

The concentration of the compounds used in the medium was in the same range as the plasma concentration of the substances after i.v. injection* [4] *in vivo*.

Release studies. The release of the various compounds from the cells is shown in Fig. 2. ICG had the lowest tendency to leave the cells: after 60 min only 13 per cent of the original amount in the cells was found in the medium, whereas in case of DBSP and APAEB after 60 min up to 81 per cent and 50 per cent was released from the cells. Also in the release experiments with DBSP a final equilibrium was reached, which allowed a pharmacokinetic analysis similar to that performed in the uptake studies. The rate constants for the release and uptake processes were found to be 0.090 min^{-1} (r_{12}) and 0.021 min^{-1} (r_{21}) in accordance with the uptake studies. The release of APAEB was linear in time, while in case of ICG no reliable rate constants could be calculated because of the short lasting first phase.

The release of drugs from the cells was also a temperature dependent process: at 0° no material was released in the medium.

* Vonk *et al.*, to be published.

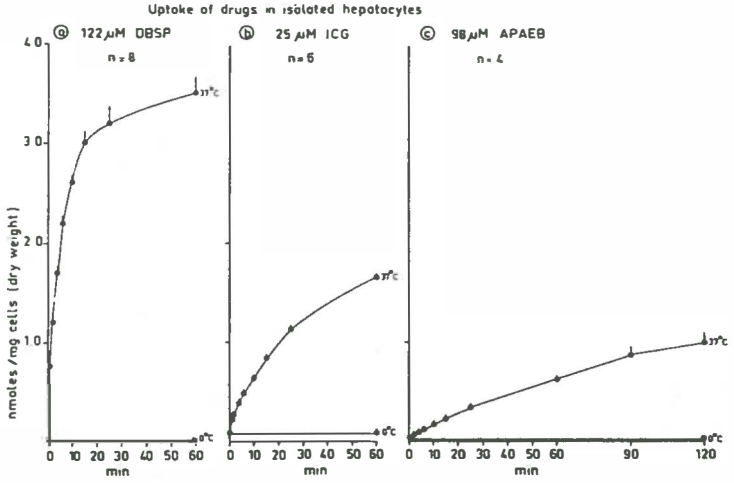

Fig. 1. Net uptake of DBSP, ICG and APAEB in isolated hepatocytes. Drug concentration in medium at $t = 0$ was 122 μM, 25 μM and 98 μM respectively.

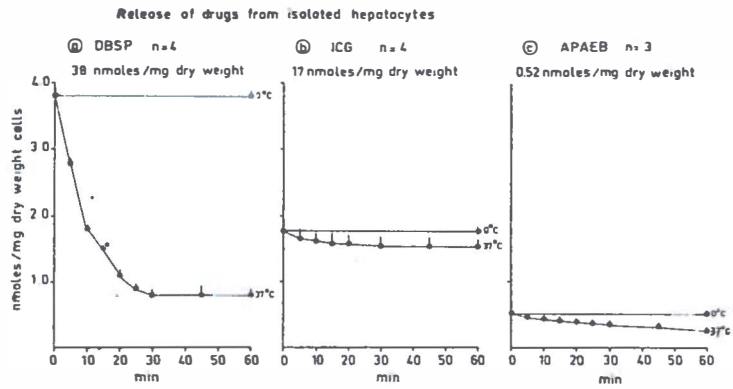

Fig. 2. Net release of DBSP, ICG and APAEB from isolated hepatocytes. Cellular drug content at $t = 0$ was 3.8, 1.7 and 0.52 nmoles/mg dry weight respectively.

Comparison in vivo-*isolated cells.* The best way to compare the transport processes *in vivo* and in isolated hepatocytes is comparison of the pharmacokinetic parameters in both situations. Unfortunately these could only be estimated for DBSP in the isolated hepatocytes. Rate constants for hepatic DBSP transport according to an open two compartment model (plasma compartment one, liver compartment two) *in vivo* were 0.58 min^{-1} (plasma-liver) and 0.118 min^{-1} (liver-bile)*, while the volume of distribution of compartment one was 13.0 ml. According to Weibel *et al.* [17] 83 per cent of liver-mass is formed by hepatocytes and 1.0 g liver = 150 × 10^6 cells. With these data the *in vivo* plasma clearance constant per 10^6 cells could be calculated: 76 × 10^{-4} ml min^{-1}. The same parameter in experiments with hepatocytes (clearance of the incubation medium: V_d = 3 ml) was 40 × 10^{-4} ml min^{-1}.

For the excretion process rate constants (fraction released from liver or cells per min.) can be directly compared. The rate constant for biliary excretion *in vivo* was 0.118 min^{-1}, while with isolated hepatocytes a rate constant for the release of 0.092 min^{-1} was found.

With regard to the other two drugs no direct comparison *in vivo*-isolated hepatocytes could be made. However, it was possible to compare the *mean clearance* by the isolated hepatocytes (ml. min) with the *mean hepatic clearance in vivo*. Clearance was calculated by dividing the total amounts of drug taken up in the liver or hepatocytes during the first 20 min peiiod per. min by the mean plasma or medium concentration in that period. Ratios between mean clearance *in vivo* and mean clearance by hepatocytes were 0.40, 0.25 and 2.23 for DBSP, ICG and APAEB respectively. Furthermore, the amounts released from the hepatocytes and biliary excretion *in vivo*, as per cent of the total amount which had been taken up in the liver, has been compared. The ratio biliary excretion-cellular release was 0.83, 0.82 and 1.27 for DBSP, ICG and APAEB respectively. Additional data concerning cytosol content and binding to cytosol proteins* were given (Table 1B), which should be considered in the explanation of differences in cellular transport of the compounds showing that ICG is much more associate with liver cell particles and cytosol proteins than DBSP and APAEB.

Influence of bile salts. The influence of bile salts on transport of the three compounds was subsequently tested. In uptake experiments (Fig. 3a) 1.0 mM taurocholate had* a distinct effect on the amount ICG present in the hepatocytes during 60 min incubation time. The effect of 1.0 mM taurocholate on the net release of ICG (Fig. 3b) was much less pronounced and even absent in the initial part of the experiment. In uptake and release experiments with DBSP similar effects of 1.0 mM taurocholate could be observed (Fig. 4). Pharmacokinetic analysis of the release curve of DBSP in presence of taurocholate revealed that r_{12} amounted to 0.090 and r_{21} was very small.

To characterize the effect of taurocholate on transport of both compounds, we studied the concentration dependency of the effect. The influence of various concentrations taurocholate on net uptake of DBSP, ICG and APAEB is shown in Fig. 5. The amount of DBSP, ICG and APAEB in the cells after 30 min incubation time with the particular drug, in presence of bile salts, expressed as percentage of the cellular content in control experiments without bile salts, is represented as a function of the log taurocholate concentration. With all three compounds used, taurocholate concentrations of 0.05–3.3 mM caused a reduction of the amount of drugs present in the cells. In all experiments the concentration, which resulted in a 50 per cent effect could be calculated (100 per cent effect was defined as the difference between 3.3 mM and 0 mM taurocholate). In the experiments with DBSP, ICG and APAEB this concentration was 0.07 mM, 0.10 mM and 0.06 mM respectively. The same experiments were performed with ICG and dehydrocholate, a concentration of 0.11 mM resulted in 50 per cent inhibition. Viability tests with Trypan blue and enzymehistochemical investigations indicated that the observed effect of bile salts was not due to cell damage. Moreover, determination of intra-

* Vonk *et al.*, to be published.

Table 1.

A. *Isolated hepatocytes* ($t = 20$)

	uptake studies	release studies
	mean Clearance (ml/min/10^6 cells)	release (%)
DBSP (122 μM)	254×10^{-4}	71.1
ICG (25 μM)	429×10^{-4}	8.8
APAEB (98 μM)	30×10^{-4}	25.6

B. *In vivo* ($t = 20$)

	mean Clearance (ml/min/10^6 cells)	bile (%)	cytosol (%)	binding to Y, Z (%)
DBSP (75 μmoles/kg)	102×10^{-4}	59.2	74	60
ICG (12.9 μmoles/kg)	108×10^{-4}	7.2	17	>95
APAEB (35.3 μmoles/kg)	67×10^{-4}	32.4	47	<4

(A) Data concerning hepatic transport of DBSP, ICG and APAEB in isolated hepatocytes during the first 20 min in uptake or release experiments. Uptake is expressed as *mean* clearance (ml/min/10^6 cells), calculated as $(\Delta Q/\Delta t)/\bar{c} = \bar{k}$ (amount taken up in the cells during 20 min expressed per min, divided by the mean medium concentration). Release is the percentage of the initial amount in the cells.

(B) Pharmacokinetic data concerning hepatic transport of DBSP, ICG and APAEB *in vivo* 20 min after i.v. injection. Uptake is expressed as *mean* clearance, calculated as $(\Delta Q/\Delta t)/\bar{c} = \bar{k}$. The amount taken up ($\Delta Q$) is the sum of the amount in the liver at $t = 20$ and the amount excreted in bile during 20 min. $\bar{c}$ is the mean plasma concentration in the 20 min period. Excretion in bile is expressed as percentage of ΔQ. The amount in the cytosol is determined after homogenization with sucrose–phosphate buffer and binding to Y and Z proteins in cytosol was determined by gelfiltration on a Sephadex G-75S column (Vonk *et al.*, to be published). The amount of drugs present in the Y and Z elution fractions is expressed as percentage of the total amount present in the supernatant applied to the column.

cellular potassium concentrations indicated that no loss of potassium occurred due to the presence of 1.0 mM taurocholate.

The effect of various taurocholate concentrations was also studied on the release of the three compounds out of the cells (Fig. 6). In experiments with ICG and APAEB, taurocholate caused hardly any effect on the amount of the drug in the cells after 30 min release time. Even with the highest taurocholate concentrations being 3.3 mM, no distinct effects could be observed. With DBSP (Fig. 6a) the situation was more complex. With 0.5 and 1.0 mM taurocholate, the amount DBSP in the cells was reduced compared with controls, while at 3.3 mM taurocholate, no change in cellular content of DBSP was detected. Assuming that also at this higher concentration

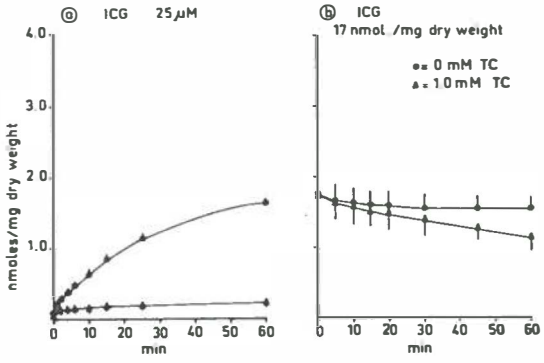

Fig. 3. Influence of taurocholate on (a) uptake of ICG ($t = 0$, 25 μM) and (b) release of ICG ($t = 0$, 1.7 nmoles/mg dry weight). ▲ = 1.0 mM taurocholate (n = 4) ● = 0 mM taurocholate.

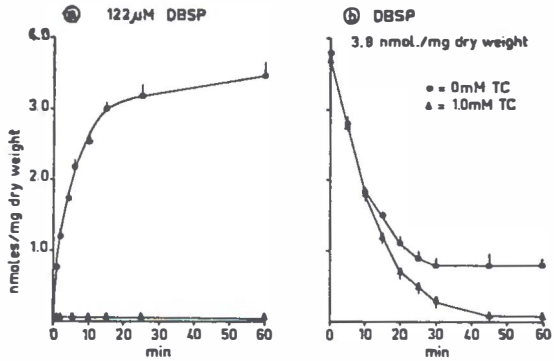

Fig. 4. Influence of taurocholate on (a) uptake of DBSP (t = 0, 122 μM) and (b) release of DBSP (t = 0, 3.8 nmoles/mg dry weight). ▲ = 1.0 mM taurocholate (n = 4) ● = 0 mM taurocholate.

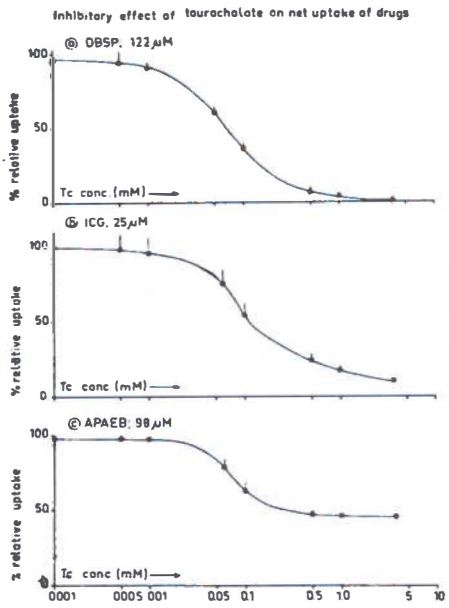

Fig. 5. Influence of various taurocholate concentrations on net hepatic uptake of (a) DBSP (t = 0, 122 μM) (b) ICG (t = 0, 25 μM) and (c) APAEB (t = 0, 98 μM). The per cent relative uptake is the cellular drug content after 30 min incubation compared to cellular drug content in control experiments without bile salts. n = 3.

taurocholate reuptake is still blocked, it follows that this is compensated for by a slower release of DBSP from the cells.

Enzymehistochemical studies. By enzymehistochemistry we studied the presence of three enzyme activities, which were reported to be localized in the plasma membrane of the hepatocyte at the bile canalicular side *in vivo*: 5'-nucleotidase, ATP-ase and leucyl-β-naphthylamidase. It was found that the first enzyme, 5'-nucleotidase showed a pattern of sharp bands on the cell surface (Fig. 7), which were branched in some cases. The ATP-ase activity showed faintly stained bands, while the leucyl-β-naphthylamidase activity could not be visualized.

In Fig. 8 the same enzyme activities are shown for liver sections, which were incubated for 30 min with

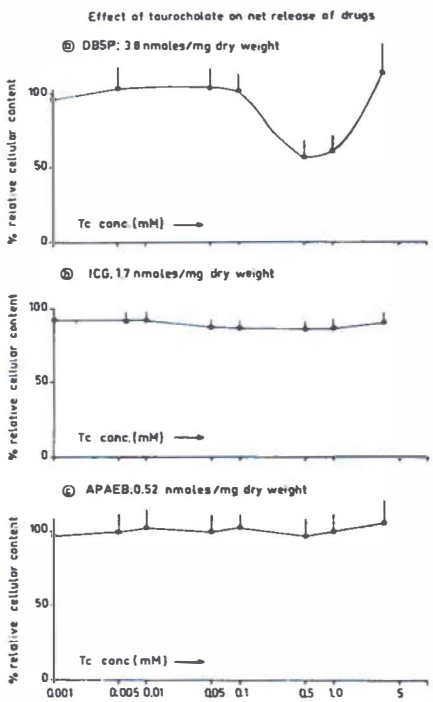

Fig. 6. Influence of various taurocholate concentrations on net release of drugs after cell loading with (a) DBSP (3.8 nmoles/mg dry weight) (b) ICG (1.7 nmoles/mg dry weight) (c) APAEB (0.52 nmoles/mg dry weight). The per cent relative cellular content is the cellular drug content after 30 min release time compared with cellular drug content in control experiments without bile salts. n = 3.

0.05% collagenase in Tris buffer. Also in this preparation the same differences between the three enzyme activity patterns could be observed. After collagenase treatment 5'-nucleotidase activity remained unchanged. ATP-ase showed a decreased activity, while leucyl-β-naphthylamidase activity was absent. This indicates that the proteolytic activity of the collagenase preparation may modify membrane bound enzymes during the isolation procedure.

DISCUSSION

Isolated hepatocytes may be a useful tool in the study of hepatic transport mechanisms. Important factors, which influence the hepatic uptake process such as bloodflow, concentration gradients between perilobular and centrolobular regions and the presence of other cell types like endothelial cells and Kupffer cells, are omitted. Several studies dealing with uptake of drugs in isolated hepatocytes were already described [18–21]. The present study confirms that isolated cells are able to take up drugs from the medium and accumulate high intracellular amounts.

Pharmacokinetic analysis of uptake experiments of DBSP provided rate constants for the uptake and release processes of 0.020 and 0.092 min^{-1} respectively. The release experiments with DBSP confirmed this result: very similar rate constants of 0.021 and 0.090 min^{-1} were found. In the presence of 1.0 mM taurocholate the rate constant for uptake was decreased to virtually zero whereas the rate constant for release was not affected (0.090 min^{-1}). This indicates that taurocholate only influenced the uptake of DBSP, but in contrast did not have any effect on the release process. The influence of taurocholate observed in the release of DBSP (see Fig. 4b) is therefore probably due to inhibition of the re-uptake, which can be observed most clearly in the last part of the experiment. Inhibition of re-uptake could not be detected with ICG and APAEB because the release of both compounds was relatively slow.

The possibility that intracellular taurocholate concentrations were too low in the first part of the release experiments to exert any effect can be ruled out, because control studies revealed that taurocholate is taken up by the cells very fast in accordance with Schwarz *et al.* [19] (in uptake studies a preincubation of 5 min with taurocholate was used).

The nature of the inhibitory effect of taurocholate on hepatic uptake of drugs is yet unclear. Detergent like effect of bile salts on hepatic plasma membranes, recently reported [8, 9] may affect the transport system across these membranes in a non-competitive manner. But dehydrocholate as well as taurocholate, having different detergent properties, show the same inhibitory potency. Another possibility could be competition between bile salts and the drug in the uptake process. However, both the uptake of the organic cation APAEB as well as the uptake of the organic anions is influenced by taurocholate with roughly the same 50 per cent effect concentration. According to Reichen and Paumgartner [22] anions like ICG and bile salts are taken up via different carriers into the hepatocytes. The inhibition of the uptake of APAEB by taurocholate, however, is not complete with the highest concentrations of taurocholate in contrast to the anions used (Fig. 5). Further studies have to be performed to elucidate the nature of the effects of bile salts on uptake of these drugs in hepatocytes.

The inhibition of hepatic uptake of drugs by high plasma concentrations of bile salts may have consequences for the *in vivo* situation. In physiological conditions the plasma concentrations of bile salts in rats is too small (about 0.01 mM) to exert any effect. Administration of small amounts of bile salts by continuous infusion (106 μmoles/hr; [10]) may have besides a stimulatory effect on biliary excretion of drugs, small inhibitory effects on hepatic uptake of the drug, because of the plasma concentration of about 0.08 mM. At high doses of bile salts inhibitory effects on hepatic uptake could be observed *in vivo* and in isolated perfused rat liver experiments.* Also in pathological conditions (cholestasis), with high plasma bile salt levels, a high retention of BSP or bilirubin may be caused by effects of bile salts on hepatic uptake of both compounds.

A disadvantage of the use of isolated hepatocytes is the difficulty establishing the direction of transport in the cells. The observed release from cells may represent the biliary excretion process as seen *in vivo* but might also reflect the transport out of the liver across the sinusoidal part of the plasma membranes. Using enzymehistochemical methods we could demonstrate that 5'-nucleotidase and ATP-ase in the isolated cells are still situated in restricted sites on the cell surface. The disappearance of the third enzyme, leucyl-β-naphthylamidase is probably caused by the collagenase treatment. The difference in enzyme activities after isolation of the cells may be caused by differences in membrane localization of the three enzymes.

The observations with 5'-nucleotidase and ATP-ase suggest the presence of "a canalicular side" of the hepatocytes; however, it remains to be proven whether this part of the membrane still participates in the excretion process. The observation that taurocholate only affects the uptake process and not the release process suggests that these processes do not occur in the same membrane region (with opposite directions). This might favour the idea that the observed release is not due to transport at sinusoidal sites, but represents the biliary excretion process.

Support for the hypothesis that the release process in isolated hepatocytes is identical to the biliary excretion process *in vivo*, can also be derived from the pharmacokinetic data. The *biliary excretion rate* of DBSP *in vivo* and the *rate of release* in isolated hepatocytes were in the same range, as well as the *clearance constants* of DBSP *in vivo* and in isolated hepatocytes. Also the rather small variations in the ratio of the *mean clearance in vivo* and in isolated hepatocytes of the three compounds and the small variations in the ratio of *mean release* in vivo and isolated hepatocytes of the three compounds (Table 1), suggest identical processes.

The comparison of the pharmacokinetic data furthermore indicate that the transport capacity of the isolated hepatocytes is preserved after the isolation procedure used in the present study.

The slow release of ICG from the hepatocytes com-

* Vonk *et al.*, to be published.

Fig. 7. Cytochemical distribution of three bile canalicular membrane-bound enzyme activities in isolated hepatocytes. (a) 5'-nucleotidase activity. Sharp tiny bands are present on the cell surface. Branches can be seen (→). Magnification was 200 times. (b) ATP-ase activity. Only diffuse bands (→) of activity are present. × 200. (c) leucyl-β-naphthylamidase. No activity can be detected. × 200.

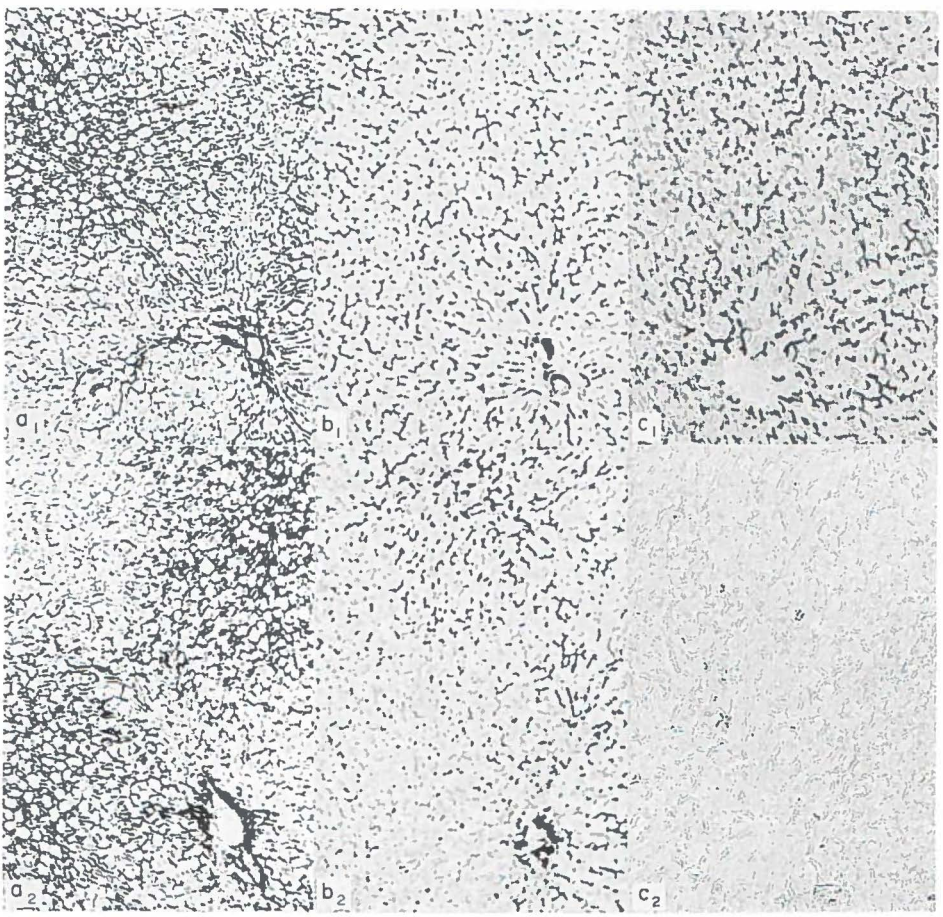

Fig. 8. Histochemical distribution of three bile canalicular membrane bound enzyme activities in liver sections treated with Tris-buffer (1) or with buffer + collagenase (2). a_1a_2 5'-nucleotidase activity: collagenase treatment does not influence enzyme activity. × 80. b_1b_2 ATP-ase activity: locally enzyme activity has been disturbed by collagenase treatment. × 80. c_1c_2 leucyl-β-naphthylamidase: enzyme activity has disappeared after collagenase treatment. × 80

pared to DBSP may be due to the low intracellular concentration of ICG (Table 1B). Most of the ICG was bound to cellorganelles, while almost 100 per cent of the amount present in cytoplasma was bound to Y and Z proteins (Table 1B). The transport maximum *in vivo* of ICG is also much lower than that of DBSP (70 and 1200 nmoles/min/kg b.w. respectively; [10]).

In our experiments no stimulatory effect of taurocholate on release of drugs could be found. The highest taurocholate concentration used in these experiments was 3.3 mM. The total concentration of taurocholate in bile *in vivo* is about 15 mM; however, the major part of bile salts in bile is sequestered in biliary micelles. The free concentration of taurocholate in bile is about 3 mM, as determined by sedimentation of biliary micelles by ultracentrifugation*, while the critical micellar concentration of taurocholate in saline has about the same value [23]. Thus, the highest taurocholate concentration used in the present experiments with isolated hepatocytes is in the same range as the free concentration of taurocholate in bile. If changes in this free concentration are responsible for the membrane effects *in vivo* resulting in an increased biliary output of organic anions, the same effects would also be expected to occur in the present experiments with isolated hepatocytes. However, such a stimulatory effect was not observed in our studies. Therefore, the hypothesis, that bile salts have a direct effect on the canalicular membrane, explaining stimulatory effects on the biliary excretion process, is not supported by these experiments.

Our study does not exclude the possibility suggested by Goresky [10], that bile salts activate hepatocytes normally not fully participating in hepatic transport of drugs, since the original lobular localization of the obtained isolated hepatocytes remains to be investigated.

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SUPPLEMENT VI

THE INFLUENCE OF BILE SALTS ON THE HEPATIC TRANSPORT OF THE ORGANIC ANION DIBROMOSULPHTHALEIN (DBSP)

ROEL VONK, MEINDERT DANHOF, THEO COENRAADS, ADRIAAN VAN DOORN, KATJA KEULEMANS, ARNOLD SCAF AND DIRK MEIJER

SUMMARY

The influence of bile salts on hepatic transport of the organic anion dibromosulphthalein (DBSP) was investigated in rats. The two main sites of interaction of bile salts with hepatic transport of DBSP, were found to be on the level of the primary hepatic uptake and the biliary excretion. The contribution of both interactions to the final effect depends on the administered dose of bile salts and DBSP.

Relatively *high doses* of bile salts inhibited hepatic uptake of DBSP.

Relatively *low doses* of bile salts stimulated bile flow and simultaneously increase maximal biliary excretion of DBSP. This stimulatory effect was observed at varying doses of DBSP, but was most pronounced at the dose of DBSP which resulted in a saturated biliary excretion. When the uncharged non-bile salt choleretic ouabain was used, also a stimulation of biliary DBSP output was found; in contrast, the anionic non-bile salt choleretics ethacrynic acid and theophylline did not stimulate biliary excretion of DBSP. Experiments performed with ^{14}C-ethacrynic acid suggested that DBSP and ^{14}C-ethacrynic acid and/or metabolites inhibited each others transport, whereas DBSP did not influence the biliary excretion of taurocholate.

Biliary transport maximum of DBSP was highly correlated with bile flow. The biliary clearance constant was only moderately changed by bile salt administration, whereas the increase in the maximal biliary excretion rate was more pronounced, suggesting an increase in V_{max} of the biliary excretion process, but also a

small increase in the apparent K_m. The most likely hypothesis to explain the increase in V_{max} is, that in case of DBSP a reversed transport from bile exists, and that the stimulation of net biliary excretion of DBSP is due to a diminished back transport from bile as the consequence of the decreased biliary concentration caused by the choleresis.

INTRODUCTION

Effects of bile salts on hepatic transport of drugs have been reported in many studies. Several authors observed a stimulatory effect of bile salts on biliary excretion of some organic anions (4, 7, 15, 18, 42, 44, 51) but also inhibitory effects of bile salts on hepatic transport were observed (6, 39, 54). To explain the stimulatory effects of bile salts on the hepatic transport process, several hypotheses have been proposed:

a. an enhanced bile flow, induced by the bile salts, stimulates biliary excretion of drugs (7, 10, 15, 42, 44)
b. bile salts per se have a specific effect on the biliary excretion process (2, 3, 4, 12, 16, 18, 20)
c. the number of hepatocytes, participating in biliary excretion of drugs is enhanced by the bile salts (21)
d. bile salts increase the rate of the hepatic uptake of drugs (36)

In this study, the influence of bile salts and other choleretics on pharmacokinetics of an organic anion, phenol-3,6-dibromphthalein disulfonate (DBSP), which is not subject to biotransformation (26, 30) was investigated in order to test the above mentioned hypotheses. Biliary excretion of DBSP was found to be a bile flow dependent process. An explanation for this phenomenon might be the existence of a reversed transport of DBSP from the biliary tree.

MATERIALS AND METHODS

Experiments in vivo

Animal experiments were performed on the same period of the day from 9.00 till 12.00 a.m., because of the circadian variation of bile flow and biliary bile salt and DBSP output (55). Male Wistar rats (280 - 310 g), which had free access to food and water, were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal^{R}) (60 mg/kg) and artificially respirated during the experiments. After an abdominal midline incision, the renal pedicles were ligated and the bile duct was cannulated with polythylene tubing. The body temperature, measured rectally, was maintained at 37.5 à 38.0^{o}C by means of a heating lamp. Via the carotid artery, blood pressure was measured in order to check the general condition of the animal and blood samples of about 150 µl were taken and collected in heparinized capillary tubes (Sherwood, Med. Ind. Inc., St. Louis, U.S.A.). Infusions (3.8 ml/h) were given via the jugular vein by means of a Braun (Melsingen, Germany) constant infusion pump. Constant infusions of choleretics were preceded by a priming dose of 0.5 ml of the infusion solution. By this procedure bile production was fairly constant, 30 min after starting the infusion. At that moment, about 45 min after cannulation of the bile duct, when output rate of endogenous bile salts was stabilized (unpublished observations) a single injection of the dye was given via the same jugular vein.

Isolated perfused rat liver experiments

Ouabain experiments were performed in the experimental setup as described by Meijer and Weitering (37). In all other experiments 100 ml erythrocyte-free perfusion medium was used, containing Krebs-bicarbonate solution and 1 % albumin. In these experiments with erythrocyte-free perfusion medium sufficient oxygen supply was provided by an enhanced perfusion rate of 35 ml/min. To replace the bile salts, physiologically originating from the enterohepatic circulation of bile salts, in all experiments an infusion of 15 µmoles/h of taurocholate was given. The viability of the isolated perfused livers was tested by measuring perfusion flow through the liver, pH of the perfusion medium and the bile flow. The latter amounted in control situations to about

12 µl/min. In some experiments, bile salts were added by a continuous infusion and 30 min after starting the infusion, DBSP was added to the medium. Bile was collected in the first forty min in 5 min fractions and afterwards in 10 min fractions; samples of the perfusion medium were taken at 2, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60, 80, 100 and 120 min before passage through the liver.

Determination of subcellular distribution

Twenty min after injection of the drug, the liver was perfused in situ via the portal vein with 50 ml saline and removed from the animal. In contrast to the method used previously (38), in the present experiments a 25 % liver homogenate in 0.01 M sucrose - 0.01 M phosphate buffer (pH 7.4) was prepared, according to procedures used for determination of dye-binding to cytosol proteins (32). The homogenate was prepared by using a Teflon-glass motor driven Potter-Elvehjem homogenator. The homogenate was centrifugated at 4°C during 120 min at 160.000 g in a Spinco ultracentrifuge. After centrifugation the supernatant and the sediment was resuspended in sucrose-phosphate buffer and samples of the supernatant and the sediment suspension were taken for determination of drug content. From the supernatant a sample of four ml was subjected to column chromatography with Sephadex S 75-S. The column (Ø 2.5 cm, h 83 cm) was eluted with 0.01 M phosphate buffer pH 7.4 (16 ml/h) at 4°C and four ml fraction was collected in which protein content (E 280) and DBSP were measured.

Calculation of cytosol concentration

Cytosol concentrations of APAEB and dTc were estimated from the supernatant concentration after ultracentrifugation of liver homogenates, according to the method described earlier (38). Some modifications of that method were introduced: the volume of the biliary tree in rat liver was assumed to be 0.32 % of the liver weight (17); 3 volumes of 0.01 M sucrose - 0.01 M phosphate

buffer for each g of liver were used instead of 1.5 volume of 0.15 M KCl; redistribution between cell organelles and cytosol during homogenisation was assumed not to occur. Drug concentration in the hepatic extracellular space was neglected, because vascular perfusion of the liver in situ with 50 ml saline will remove most of the extracellular drug, assuming that the exchange of drug between plasma and extracellular space outside plasma is sufficiently rapid. Cytosolic concentration was calculated in this case from the expression:

$C_{cyt} = 8.098\ C_{sup} - 0.007\ C_{bile}$. (derived from calculations of Meijer et al. (38).

Binding of drugs to biliary micelles

Relative binding of drugs to biliary micelles was studied by means of ultracentrifugation of bile, according to the procedure previously described (52). DBSP was added to bile in vitro to a concentration of 9.2 mM. Binding of various bile salts to biliary micelles in bile, obtained after infusion of 106 µmoles/h of taurocholate or dehydrocholate, was also measured. Bile was collected during two hours from several rats, which received the particulate bile salt infusion, was subsequently pooled and centrifugated.

Chemical compounds

The chemicals used were obtained from the following sources: DBSP: Société d'Etudes et de Recherches Biologiques (SERB), Paris, France; ouabain (g-strophantin): Merck A.G. Darmstadt, Germany; sodium dehydrocholate and sodium taurocholate: Fluka A.G., Buchs, Switzerland; theophylline, cholate, glycocholate, taurodeoxycholate, taurodehydrocholate and glycodehydrocholate: Calbiochem (San Diego, Cal., U.S.A.); ethacrynic acid and ^{14}C-labelled ethacrynic acid was donated by Merck, Sharp & Dohme, Haarlem, The Netherlands; ^{3}H-taurocholic acid: New England Nuclear Inc., Boston, U.S.A.

Chemical analysis

DBSP was determined in bile and plasma. 50 µl plasma was diluted with 5 ml 0.1 N NaOH and measured spectrophotometrically at 580 nm. 10 µl of bile was diluted with 10 or 20 ml 0.1 N NaOH and measured in the same way. After injection of DBSP, bile samples were subjected to thin layer chromatography in order to study possible chemical modifications. Thin layer chromatography with Silicagel 60 F (Merck A.G.) and n-butanol, acetic acid and distilled water (4 : 1 : 2) did not indicate that DBSP was metabolized, which is in accordance with Javitt (26) and Klaassen & Plaa (30), but in contrast with Javitt (27). In the latter report, 3-6 dibromosulphthalein has shown to be (partly) conjugated with glucuronic acid in Gunn rats.

Bile acid and cholesterol were determined according to the method of Roovers, Evrard & Vanderhaeghe (45) and Van Berge Henegouwen, Ruben & Brandt (48) with minor modifications. After methylation, the bile acids were chromatographed as their trifluoroacetate derivatives on a column of 1 % OV-210 on Cromosorb W-HP 100-120 mesh and analyzed by flame ionization in a Packard-Becker Model 419 gaschromatograph. Combined gas-liquid chromatography/mass spectrometry was carried out on the trifluoroacetate derivatives. A Varian Mat 112 mass spectrometer at 70 eV with an accelerating voltage of 820 V and a scan time of 200 mass units/sec was used.

Radiochemical analysis

^{3}H-taurocholate and ^{14}C-ethacrynic acid were estimated with a liquid scintillation spectrometer (Nuclear Chicago Mark II). Plasma and bile samples were dissolved in 10 ml of aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.). The samples were counted at an efficiency of about 85 % for ^{14}C and 35 % in case of ^{3}H. Quencing of each sample was corrected for by external standardization.

Pharmacokinetic analysis

Intrinsic clearance of DBSP in isolated perfusion experiments was calculated from the mean hepatic clearance using the equation

$$Cl_H = F \times \frac{Cl_i}{F + Cl_i} \quad (57)$$

F: perfusion flow through the liver; Cl_H: mean hepatic clearance, which was calculated from the dose and area under the plasma disappearance curve (57). Since perfusate flow was constant during the perfusion experiments (35 ml/min), Cl_i (intrinsic clearance) could be calculated. The latter parameter describes clearance due to the biliary excretion (ml/min). This process can also be conceptualized by the V_{max}/K_m ratio (57).

Statistical analysis

Statistical comparisons were made using Wilcoxon test. Unless specified otherwise, the term "significant" means a p value of less than 0.05.

RESULTS

In vivo experiments, the effect of bile salts

Biliary excretion of DBSP in vivo was measured after injection of 2.5, 75 and 150 µmoles/kg body wt. (Fig. 1). The maximal biliary excretion rate (Tm), obtained with the latter dose, was 1.74 µmoles/min/kg body wt. Administration of 106 µmoles/h of dehydrocholate stimulated biliary excretion of DBSP. This stimulatory effect was dependent on the dose of DBSP used: the increase in the top of the excretion curve was most pronounced with a dose of 150 µmoles/kg, while the increase in the top with 2.5 µmoles/kg was small. In case of the latter dose, the semilogarithmically plotted descending phase of the biliary excretion curve was linear and the slope could be calculated. In the control experiments the mean half life was 5.7 min, while during dehydrocholate administration it was only slightly lower.

The dose of 150 µmoles/kg body wt. was used to investigate the influence of a series of different bile salts on hepatic

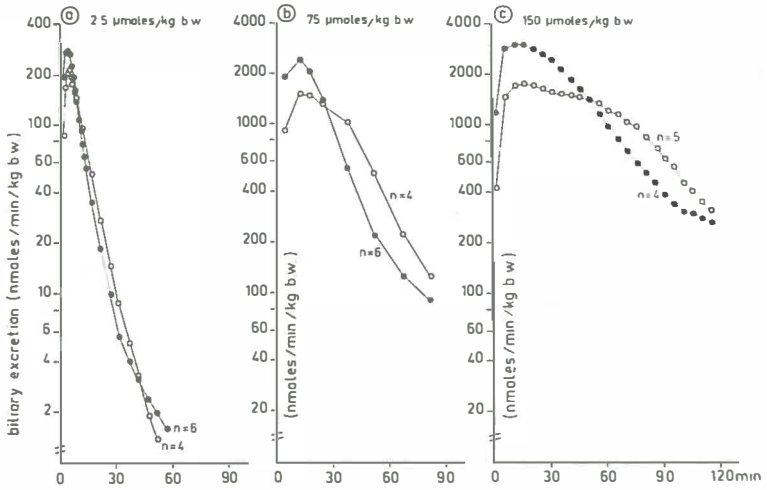

Fig. 1. Biliary excretion of DBSP (nmoles/min/kg body wt.) in vivo after injection of DBSP a. 2.5 µmoles/kg; b. 75 µmoles/kg; and c. 150 µmoles/kg during infusion of saline (o—o) and 106 µmoles/h of dehydrocholate (●—●). Mean values S.E. amounted to maximal 7 %.

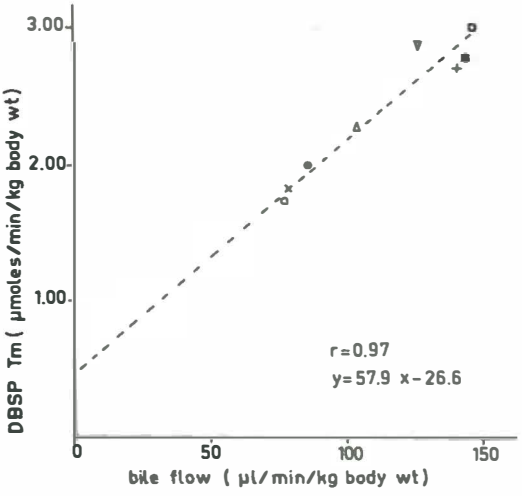

Fig. 2. In vivo correlation between transport maximum of DBSP (DBSP Tm) and bile flow after injection of 150 µmoles/kg of DBSP during administration of saline (o), administration of 106 µmoles/h of cholate (▽), taurocholate (△), glycocholate (●), dehydrocholate (□), taurodehydrocholate (■), glycodehydrocholate (+) or 25 µmoles/h of taurodeoxycholate (x). Each point was the mean value of four experiments.

transport of DBSP. In Fig. 2 DBSP Tm in various experiments was plotted versus bile flow, induced by administration of 106 µmoles/h of cholate, taurocholate, glycocholate, dehydrocholate, taurodehydrocholate, glycodehydrocholate or 25 µmoles/h of taurodeoxycholate respectively. Taurodeoxycholate was administered in a lower dose, because high doses of this bile salt result in haemolysis and general toxic effects in the animals. The bile flow, plotted in Fig. 2 represents the mean value observed at maximal biliary DBSP excretion. A high correlation (r = 0.96) between Tm and bile flow was found. Plasma disappearance of DBSP, however, was only slightly changed by these doses of bile salts. The mean half lives of the first phase of the biexponential disappearance curves were not altered by infusion of bile salts, whereas the half lives of the second phase of the curves were maximally 20 per cent lowered compared with the control studies.

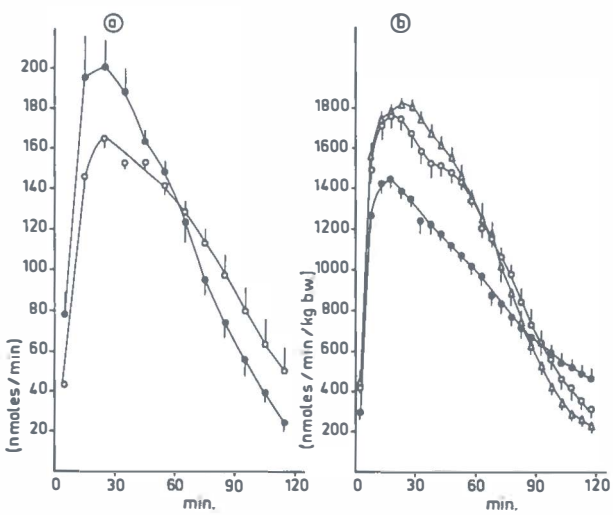

Fig. 3. Biliary excretion of DBSP during administration of non bile salt choleretics

a. in isolated perfused rat liver experiments during infusion of saline (o——o), or 31.6 µmoles/h of ouabain (●——●) after addition of 14.7 µmoles of DBSP; n = 4

b. in vivo during infusion of saline (o——o) (n = 5) or infusion of 63 µmoles/h of ethacrynic acid (●——●) (n = 5) or injection of 70.5 µmoles of theophylline (Δ——Δ) (n = 4) after injection of 150 µmoles/kg of DBSP. Mean values ± S.E.

The effects of non-bile salt choleretics ethacrynic acid and theophylline

In experiments in vivo, when theophylline and ethacrynic acid were used as choleretics, no stimulation of biliary output of DBSP was observed (Fig. 3b). With ethacrynic acid, in spite of a distinct elevation of bile flow, the biliary excretion of DBSP was even decreased. Theophylline stimulated bile flow, but failed to induce a significant stimulation of DBSP excretion. Both theophylline and ethacrynic acid are excreted in bile as organic anions, most likely as glucurinide and glutathion conjugates respectively (31, 33).

To study the possible competition of some of the choleretics with DBSP in the biliary excretion process, the influence of DBSP on biliary excretion of ^{14}C-ethacrynic acid and/or metabolites and on ^{3}H-taurocholate was investigated. Fig. 4 shows that DBSP

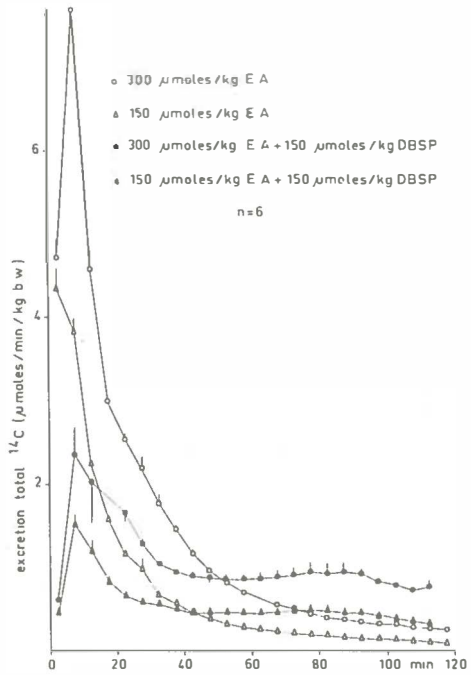

Fig. 4. Biliary excretion of ^{14}C-ethacrynic acid and/or metabolites (µmoles/min/kg body wt.) after injection of ^{14}C-ethacrynic acid with addition of saline (o, Δ) or DBSP (•, ▲).

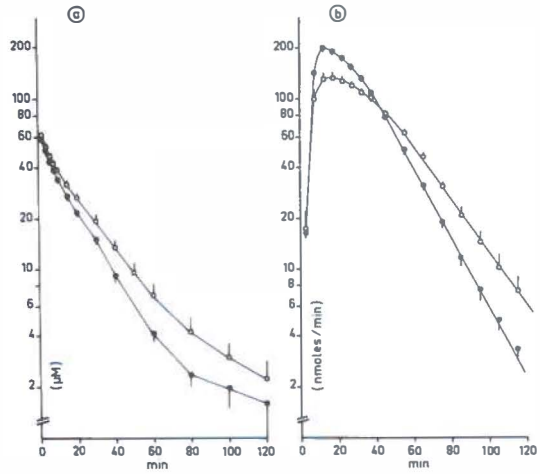

Fig. 5. Medium concentration and biliary excretion rate of DBSP in isolated perfused rat liver experiments after addition of 7.4 μmoles of DBSP during infusion of saline (o——o), 45 μmoles/h of taurocholate (●——●). Mean value ± S.E.; n = 4.

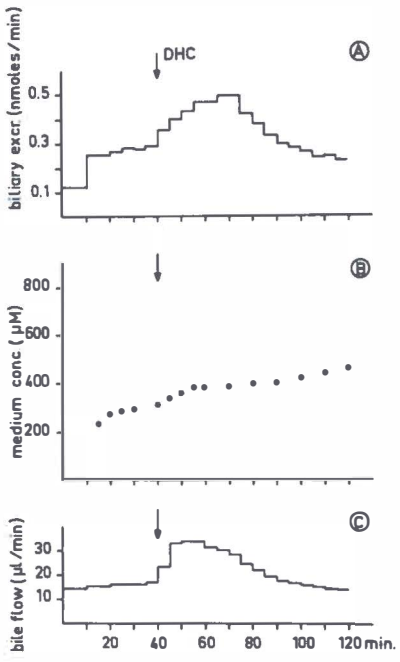

Fig. 6. Biliary excretion rate, medium concentration and bile flow in an isolated perfused rat liver experiment after continuous infusion of DBSP (0.57 μmoles/min). Dehydrocholate (DHC) was given in a single dose of 50 μmoles.

inhibits hepatic transport of ^{14}C-ethacrynic acid and/or metabolites. With ^{3}H-taurocholate similar experiments were performed. Hepatic transport of ^{3}H-taurocholate, after injection of 37.5 μmoles/kg was not inhibited by administration of 150 μmoles/kg of DBSP.

Liver perfusion experiments

The effects of bile salts on DBSP elimination in vivo might be influenced by indirect effects such as changes in distribution volume and blood flow through the liver. Therefore we investigated the influence of bile salts in isolated perfused rat liver experiments, in which plasma flow was standardized and extra hepatic interactions were minimized. DBSP was administered in separate series of experiments by bolus injection or continuous infusion. Fig. 5 shows medium concentrations and biliary excretion of DBSP after administration of a bolus injection of 7.4 μmoles of DBSP. An increased bile flow was induced by 60 μmoles/h of taurocholate, being four times the dose administered in the control experiments. The disappearance of DBSP from the perfusion medium was slightly accelerated by taurocholate administration. Maximal biliary excretion of DBSP was increased with 54 %, whereas the half life of the descending phase of the biliary excretion curve was only increased with 30 % by the additive taurocholate infusion. Mean hepatic clearance was 4.4 and 5.7 ml/min and the intrinsic clearance 5.0 and 6.8 ml/min in control studies and during taurocholate administration respectively. An increased maximal biliary excretion of DBSP was also observed after a continuous infusion of DBSP and administration of 50 μmoles of dehydrocholate by single injection. An infusion rate of 5.7 μmoles/min of DBSP was used, which was about twice the maximal biliary excretion rate in isolated perfused livers, resulting in a rather constant biliary excretion and rising plasma concentrations of DBSP. In Fig. 6 the biliary excretion rate, the medium concentration of DBSP and the bile flow are shown. Both bile flow and biliary DBSP excretion were stimulated by the addition of dehydrocholate. The rise in the perfusion

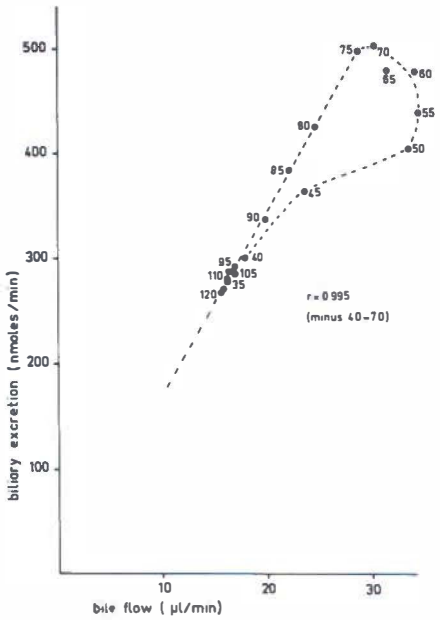

Fig. 7. Correlation of bile flow (µl/min) and biliary excretion rate of DBSP (µmoles/min) in isolated, perfused liver experiments. DBSP was administered in a continuous infusion of 0.57 µmoles/min and 40 min after the start of the infusion of DBSP, 50 µmoles of dehydrocholate were given. The numbers indicate the time course after starting the DBSP infusion.

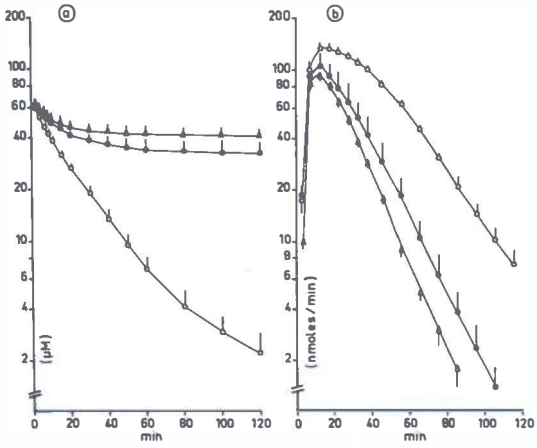

Fig. 8. Medium concentration and biliary excretion rate of DBSP in isolated perfused rat liver experiments after addition of 7.4 µmoles of DBSP during infusion of saline (o——o), 106 µmoles/h of dehydrocholate (●——●) and 159 µmoles/h of taurocholate (Δ——Δ). Mean value ± S.E.; n = 4.

medium concentration of DBSP was initially accelerated by dehydrocholate, but after that it clearly decreased. Relating bile flow to biliary excretion of DBSP in these experiments, a high correlation was found ($r = 0.99$), but only if the 30 min period following addition of dehydrocholate (40-70 min) was excluded (Fig. 7). In this period biliary output of DBSP was much less increased than bile flow, especially in the first twenty min after injection of the bile salt. This phenomenon gradually disappeared in the course of the experiment.

The experimental setup of the isolated perfused liver enabled us to study the influence of high doses of ouabain on DBSP kinetics. Earlier studies (22, 41) showed, that moderate doses ouabain can induce a profound choleresis in isolated perfused livers. Ouabain was administered by a continuous infusion of 31.6 µmoles/h, resulting in a 30 % increase in bile flow compared with control experiments. Fig. 3a shows an increase of the maximal biliary excretion of DBSP of about 25 % due to ouabain choleresis and a somewhat steeper slope of the biliary excretion curve.

Inhibitory effects of bile salts in liver perfusion

Since the single injection experiments with dehydrocholate (Fig. 7) suggested an additional effect of the bile salt immediately after the injection, the effect of relatively high doses of bile salts on hepatic transport was studied in the perfusion setup. In Fig. 8 the influence of two bile salts, taurocholate (159 µmoles/h; slightly above the biliary transport maximum for taurocholate (39)) and dehydrocholate (106 µmoles/h) on pharmacokinetics of DBSP is shown. Besides the first 10 minutes of the experiments, medium disappearance of DBSP was clearly inhibited by both bile salts, while maximal biliary excretion was not stimulated in this case. The slopes of the biliary excretion curves, however, were slightly steeper in comparison with the control experiments. In similar experiments the medium concentration of ^{3}H-taurocholate during infusion of 159 µmoles/h was measured. In Fig. 9 the disappearance of DBSP from the perfusion

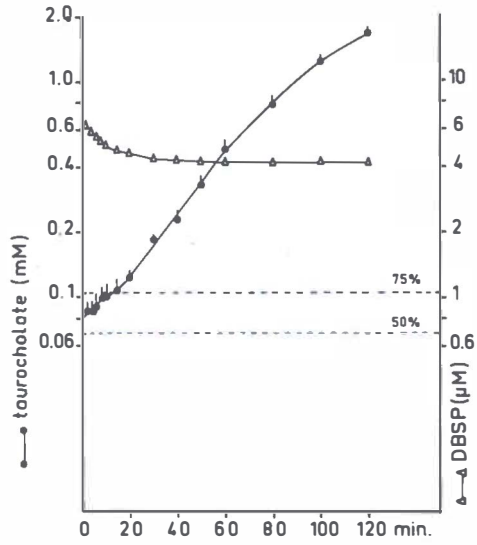

Fig. 9. The concentration of DBSP (Δ——Δ) and ^{3}H-taurocholate (●——●) in the medium compartment of isolated perfused rat livers after addition of 7.4 μmoles of DBSP and infusion of 159 μmoles/h of ^{3}H-taurocholate. The broken lines indicate the 50 % and 75 % inhibitory taurocholate concentrations for hepatic uptake of DBSP in isolated hepatocytes as reported previously (53).

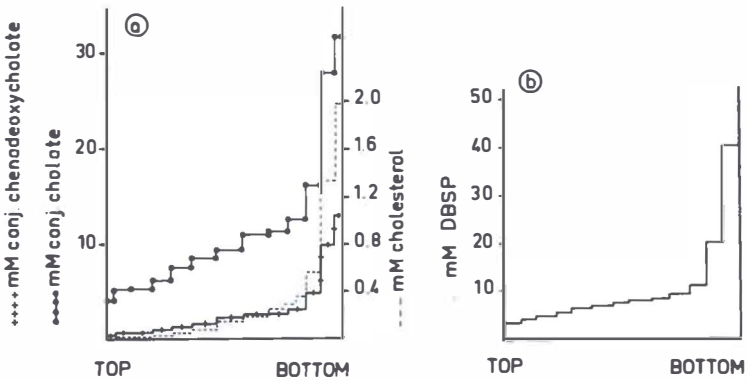

Fig. 10. Sedimentation patterns of bile after ultracentrifugation (17 h, 162.000 g_{av}).

a. bile collected from control rats

b. bile collected from control rats, to which DBSP was added in vitro (9.2 mM)

medium during taurocholate infusion and the medium concentration of ^{3}H-taurocholate are shown, clearly indicating progressive accumulation of ^{3}H-taurocholate in the medium. Within 20 minutes of infusion of taurocholate, bile salt concentrations were reached, which in earlier studies (53) were shown to cause a 75 % inhibition of DBSP uptake in the hepatocytes.

Subcellular distribution of DBSP

Binding of DBSP to the particulate and supernatant fractions of liver homogenates, after injection in vivo, was studied to investigate the influence of bile salts on intracellular distribution. The amount in the liver, 20 min after injection, was decreased by bile salts, as well as the calculated DBSP cytosol concentration (Table I). The distribution ratio cytosol/particles seemed to be increased by bile salt administration.

Composition of bile during bile salt infusion

To get more information on the composition of bile during infusion of bile salts in relation to the observed stimulatory effects on biliary excretion of DBSP, bile salt concentration and composition as well as micellar binding of DBSP and some of the bile salts were studied. Bile was collected during two hours from several rats and pooled before ultracentrifugation and subsequent gaschromatographic analysis. In Fig. 10 the sedimentation patterns of DBSP and of three endogenous biliary components are shown. Under the centrifugation conditions chosen in the present experiments, the sedimentation pattern indicated, that 65 % of DBSP is associated with cholesterol containing micelles. In Table II the composition of bile after infusion of 106 µmoles/h of taurocholate or dehydrocholate is shown. By ultracentrifugation "free" concentration and % bound to micelles were calculated according to the method described previously (52). Control studies indicated, that the mean bile salt concentration in pooled bile from male rats, collected from 30 min to 120 min after cannulation of the bile duct, was 9.6 mM, of which

TABLE I

	saline	taurocholate (106 μmoles/h)	dehydrocholate (106 μmoles/h)
% liver	24.5 ± 0.8	14.1 ± 0.9*	15.3 ± 1.7*
conc. cytosol (mM)	0.66 ± 0.06	0.46 ± 0.04*	0.34 ± 0.01*
% bound to particles	57 ± 2	51 ± 1*	48 ± 3*
ratio cytosol/particles	0.76 ± 0.05	0.97 ± 0.04*	1.10 ± 0.12*

Table I. Hepatic content, concentration in liver cytosol and hepatocellular distribution ratio of DBSP twenty minutes after injection in vivo of 75 μmoles/kg of DBSP during infusion of saline, taurocholate (106 μmoles/h) and dehydrocholate (106 μmoles/h). The amount in the liver is expressed as percentage of the dose; the amount bound to particles is expressed as percentage of the amount in the liver.

TABLE II

	infusion taurocholate (106 μmoles/h)			infusion dehydrocholate (106 μmoles/h)		
Compound	conc. (mM)	"free" conc. (mM)	% bound to micelles	conc. (mM)	"free" conc. (mM)	% bound to micelles
cholate	49.10 ± 0.50	16.22 ± 0.61	67 ± 2	6.04 ± 0.10	3.81 ± 0.03	36 ± 1
chenodeoxycholate	0.92 ± 0.01	0.16 ± 0.05	83 ± 5	0.47 ± 0.02	0.15 ± 0.02	67 ± 6
deoxycholate	0.64 ± 0.00	0.14 ± 0.06	78 ± 9	0.30 ± 0.05	0.10 ± 0.03	67 ± 6
lithocholate	0.05 ± 0.02	–	–	0.09 ± 0.00	–	–
cholesterol	0.22 ± 0.02	0.03 ± 0.01	85 ± 6	0.06 ± 0.01	< 0.01	> 85
dehydrocholate				–	–	–
7,12-diketolithocholate				13.14 ± 2.03	9.82 ± 1.01	24 ± 4
3,7-dihydroxy-12-ketocholate				2.88 ± 0.44	1.95 ± 0.18	32 ± 6
7-ketodeoxycholate				17.82 ± 0.51	13.71 ± 0.52	23 ± 2
total bile salts	50.7	16.5	67	40.8	29.5	40
	total bile flow 1.99 ml/h/animal			total bile flow 2.65 ml/h/animal		

Table II. Biliary concentration of various components after infusion of taurocholate (106 μmoles/h) or dehydrocholate (106 μmoles/h). Bile was collected during two hours from several animals and pooled before analysis. Bile was ultracentrifugated (17 h, 162.000 g_{av}) and the "free" concentration and the percentage bound to biliary micelles subsequently determined. Mean values ± S.E. n = 3.

about 90 % was conjugated cholate. Infusion of 106 μmoles/h of taurocholate and dehydrocholate resulted in a five fold increase in total bile salt concentration (Table II). Total bile flow in taurocholate and dehydrocholate experiments was increased with 88 per cent and 150 per cent respectively. Total bile salt output/h was about ten times higher during bile salt infusion in comparison with bile salt output/h in control experiments. Dehydrocholate was almost completely metabolized, while the formed metabolites, identified by mass spectrometry, had a low tendency to form biliary micelles, in accordance with other studies (14, 19, 24, 47). Since in control bile the "free" concentration of bile salts is about 3.3 mM, bile salt infusion resulted in a considerable increase in the "free" bile salt concentration.

DISCUSSION

The hepatic transport of DBSP is characterized by a fast hepatic uptake and biliary excretion of the compound. Two hours after injection in vivo 85 per cent of a dose of 150 μmoles/kg body wt. was excreted in bile in the unchanged form. Total plasma, cytosol and bile concentration twenty min after injection of the compound were 0.12, 0.66 and 18.9 mM respectively. After correction for binding to plasma, cytosolic proteins (40) and binding to biliary micelles, considerable concentration gradients between bile, cytosol plasma were obtained for free drug.

Since large amounts of DBSP were found to be bound to the particulate fraction of liver homogenates, the determined cytosol concentration might be an overestimation due to homogenization artifacts. This would imply, that the bile/cytosol concentration ratio would even be higher than it was calculated, but it remains uncertain, whether there is real uphill transport from plasma to the cytosol compartment of the liver. Thus, the biliary excretion process probably is of an active nature and consequently various kinds of interactions have to be expected.

The binding capacity of the cell organelles seems far to exceed the binding capacity of ligandin and Z protein (Table I), which was also reported in case of ICG (49). Interactions of

drugs on the level of binding to cell organelles therefore, may have implications for the hepatic transport of drugs.

Conflicting results concerning the influence of bile salts on hepatic transport of drugs have been reported. In some cases administration of bile salts stimulated biliary excretion of drugs, but also inhibitory effects or lack of effect were reported (see Introduction). In our study concerning the organic anion DBSP, the same variations in effects were observed: stimulatory, inhibitory or lack of effects of bile salts depending on the experimental conditions.

The stimulation of bile flow and biliary excretion of drugs by bile salt administration in vivo is not due to indirect effects such as influences on blood flow and blood pressure, because also in perfused rat livers under (perfusion medium) flow standardized conditions these stimulatory effects were observed.

The results indicate that the degree, in which dehydrocholate stimulates DBSP excretion, is dependent on the dose of DBSP used. If DBSP is administered in amounts, which results in a Tm for biliary excretion (bolus injection or continuous infusion), a pronounced effect on this Tm caused by bile salt administration was found. When a small dose of DBSP was used, which causes a maximal biliary excretion rate far below Tm (about 0.1 x Tm), only a slight increase in the top of the biliary excretion curve was found. The highest dose of DBSP, resulting in a maximal biliary excretion rate equal to the Tm value, was used to study the influence of various bile salts. Bile salts with different properties were used: bile salts with a relatively low choleretic effect, together with a strong micelle forming ability and detergent potency (taurodeoxycholate, taurocholate, cholate and glycocholate), and bile salts with a high choleretic effect and a weak micelle forming capacity (dehydrocholate, taurodehydrocholate and glycodehydrocholate).

Composition of bile was analyzed in more detail after infusion of 106 μmoles/h of taurocholate and after infusion of 106 μmoles/h of dehydrocholate. Table II shows that the total bile salt concentration and the percentage of bile salts, incorporated into cholesterol containing biliary micelles is lower

after dehydrocholate administration, 50.7 and 40.8 mM respectively, whereas this value amounted to 9.6 mM in control situations. The biliary output of bile salts was 10, 101 and 108 μmoles/h/animal after saline, taurocholate and dehydrocholate administration. The "free" concentration of bile salts was distinctly higher after dehydrocholate administration, than during taurocholate administration, thus the increase in "free" bile salt output did not result in a comparable increase in bile flow. The amount cholesterol excreted in bile was significantly lower during dehydrocholate infusion than during taurocholate infusion, which is in agreement with other observations (25). Dehydrocholate was almost completely metabolized, and its metabolites were poor micelle formers (Table II), which confirmed earlier reports (14, 19, 24, 47).

When DBSP Tm and bile flow were correlated in experiments, in which various bile salts were administered, a high correlation coefficient was found. Within individual experiments with isolated perfused livers, when DBSP was administered by continuous infusion and dehydrocholate by bolus injection, a high correlation was found between bile flow and biliary excretion rate of DBSP, in the last part of the experiment. In the first part of the experiment, immediately after dehydrocholate administration, additional effects of bile salts occurred, which will be discussed later on. These high correlations suggest, that bile salt induced bile flow per se is an important factor in the biliary excretion process of DBSP.

If bile flow itself regulates biliary excretion of DBSP, non-bile salt induced choleresis should also stimulate biliary excretion of DBSP. Therefore, the influence of three non-bile salt choleretics was investigated, ouabain, an uncharged compound whose biliary excretion is not affected by DBSP (unpublished results) and theophylline and ethacrynic acid, which both are metabolized and excreted in bile as organic anions (31, 33). Ouabain administration caused an increase in biliary excretion of DBSP, whereas ethacrynic acid and theophylline did not, in spite of a distinct choleresis. The lack of a stimulatory effect on

biliary DBSP excretion of both anionic non-bile salt choleretics could be explained by the assumption that the stimulatory effect on biliary excretion of DBSP in principle is present, but masked by competitive inhibition in hepatic transport between DBSP and both choleretic agents.

To test this hypothesis the influence of DBSP on hepatic transport of ^{14}C-ethacrynic acid was investigated. Our experimental data indicate, that DBSP inhibits the biliary excretion of ^{14}C-ethacrynic acid and/or metabolites. The relative amounts of ethacrynic acid and metabolites in bile, however, were not markedly changed, thus inhibition of the conjugation process of ethacrynic acid by DBSP does not form a likely explanation for the interaction. Moreover, the biliary excretion of the ethacrynic acid-glutathion conjugate was also inhibited by administration of DBSP. Indirect evidence for the interaction between DBSP and ethacrynic acid was obtained from the observation that the ethacrynic acid induced bile flow was inhibited by DBSP administration.

Similar experiments were performed with taurocholate and DBSP. When ^{3}H-taurocholate and DBSP were administered in molar ratio of 1 : 4 no inhibition of ^{3}H-taurocholate output was found. These observations confirm conclusions from earlier reports (8, 35), that not all organic anions share a common step in the hepatic transport process. The phenomenon of an intact bile salt excretion and a deficient organic anion output, which occurs in the Dubin Johnson syndrome (23) and the Corriedale sheep (1) leads to the same conclusion. These data can be explained by several carriers for organic ions or by differentiation in hepatocytes concerned with the hepatic transport of the various organic anions. Such separate transport systems for bile salts and other organic anions may have a physiological advantage, because the hepatic transport of bile salts in that case will not be influenced by changes in hepatic supply of other endogenous or exogenous organic anions. Thus, in case of ethacrynic acid competitive inhibition of DBSP excretion is likely. On the other hand ouabain may be excreted via another biliary excretion pathway, thus, in this case, the stimulatory effect due to an

increased bile flow is not counteracted by a competitive inhibition with DBSP.

Generally, an increased biliary excretion rate of a drug can be explained by an increase in V_{max}/K_m ratio of the biliary excretion process for the particular drug. An increased (apparent) V_{max} theoretically can be caused by

a. an increased dissociation of the drug-carrier complex in the bile canaliculi due to a decreased drug concentration, caused by the choleresis or increased micellar sequestration (42). The assumption is made that the carrier-drug complex is unable to move from the canalicular side back to the internal side of the canalicular membrane.
b. a reduced reversed transport of drugs from bile. This decreased reversed transport occurs by a lower drug concentration due to choleresis or high micellar binding. This reversed transport could be located on the same carrier as for transport from liver to bile, or on other carriers.
c. an allosteric interaction (18), detergent effect or co-transport which facilitates the transfer of the drug-carrier complex through the membrane.
d. an increase in the number of carriers participating in biliary transport, which may be caused by an increase in the number of cells (21) participating in the transport or an increase in the number of carriers per cel.

The results from the present study, showing that dehydrocholate is more potent in stimulating DBSP excretion than taurocholate, as well as other data reported (12, 51, 52) rules out the proposed micellar sequestration (5, 20, 56) as a determining factor in the biliary excretion process.

The observation, that the bile salts with the lowest detergent activity are the most potent in stimulating the biliary excretion of DBSP argues against membrane detergent effects as a factor; also our previous studies with isolated hepatocytes (53) do not support this hypothesis.

If allosteric interactions, as proposed by Forker (18) would be of importance, it is difficult to explain that the equal biliary bile salt flux in dehydrocholate or taurocholate infusion

experiments would lead to such unequal effects on biliary DBSP output as actually observed. The same argument makes co-transport of bile salts and DBSP to bile an unlikely explanation.

It cannot be completely ruled out, that a facilitation of the dissociation of the drug from the carrier in the canaliculus plays a role. However, if the canalicular transport step is active in the classical sense, one would expect uphill transport not to be affected by the canalicular concentration.

The mechanism which remains attractive is that reversed transport from the biliary tree occurs. Not only the presented data and data previously published (7, 10, 15, 42, 44) shows a high correlation between bile flow and DBSP Tm, but also retrograde biliary injection studies (9, 11, 43) indicate, that reversed transport from bile takes place.

However, it was reported that a number of non-bile salt choleretics do not stimulate the biliary excretion of BSP in the dog in contrast to bile salts: 4-methyl umbelliferone (16), glucagon (2), theophylline (2, 16), hydrocortison (34), SC 2644 (20) and buculome (29). This was confirmed for ethacrynic acid and theophylline with regard to DBSP in the present study.

Since most of the mentioned compounds are excreted in bile as organic anions, competitive inhibition may mask stimulatory effects. However, it is difficult to accept, that in all these studies a supposed stimulatory effect would be exactly counteracted by competitive inhibitory effects, taking into account that various dosage regimes were used. Other possibilities to explain the lack of effect of the anionic non-bile salt choleretics are the hypotheses, that the particulate choleretics may induce choleresis in hepatocytes in the liver lubule not participating in dye transport (28) or that the non-bile salt induced choleresis in the dog is not of canalicular origin in spite of an enhanced biliary mannitol clearance. The latter hypothesis may be supported by the observation, that some of the non-bile salt choleretics which were used in the dog, are not potent in the rat. It is known, that ductular modification of bile flow in this species is small (46). Thus, the failure of some non-bile salt choleretics to stimulate biliary excretion of DBSP is not necessarily in

conflict with our concept of a bile flow dependent biliary excretion mechanism of DBSP.

It should be emphasized that the findings with DBSP or BSP do not afford a general rule concerning the interaction of bile salts and organic anions. Our previous studies with ICG in the rat (50) showed that biliary output of the dye was more stimulated by taurocholate than by dehydrocholate, in spite of a higher bile flow by the latter compound. The same phenomena were found with Rose Bengal (unpublished observations). These data indicate, that interaction between choleretics and cholephylic dyes also depends on the dye used (35).

The hypothesis, that bile salts activate more centrally located hepatocytes in the liver lobule to excrete more drug (21), remains a possible mechanism, although it should be emphasized that this hypothesis is not necessarily in contrast with a bile flow dependent mechanism.

Our in vivo studies as well as the perfusion experiments strongly suggest, that the V_{max} of the biliary excretion of DBSP is increased. If V_{max} is clearly increased and the apparent K_m unchanged, one would expect a parallel increase in intrinsic clearance and a change in the terminal phase of the biliary excretion curve. However, there were only slight changes in these parameters. This might imply, that the apparent K_m was also increased by bile salt administration. A similar conclusion was reached by Forker (18) from experiments in a completely different setup. The reason for such a change in *apparent* K_m is unclear, but it could be caused by competition, some kind of competition of bile salts with DBSP, membrane modifying effects or changes in the hepatic distribution volume. The data in Table I might indicate, that there is some displacement of DBSP from particulate fraction to cytosol. We did not find evidence for competition between DBSP and taurocholate for ligandin and Z protein. The increased cytosol/particles ratio, however, could also alternatively be explained by the lower hepatic content of DBSP in the bile salt infusion studies, assuming a non linear binding in the particular concentration range.

A third level of interaction of bile salts was found on the

hepatic uptake level. Our studies indicate an inhibition of hepatic uptake of DBSP, which confirms our previous studies with isolated hepatocytes (53). The inhibitory effects of dehydrocholate were observed with lower infusion rates than in case of taurocholate. This may be due to the differences in hepatic clearance of both bile salts. If the clearance is low, the cumulation of the bile salts in the medium (plasma) will be higher and inhibitory medium (plasma) concentrations will be reached faster. Such an effect is suggested by the data presented in Fig. 9. The initial disappearance of DBSP from the medium is moderately affected and the biliary excretion curve shows that the amount of DBSP, which is taken up initially is excreted with a rate at least equal to the control experiments. But subsequently the inhibitory effect progresses in time so that almost no DBSP is taken up into the liver anymore. Dehydrocholate administration in isolated perfused liver experiments was more effective than in vivo, which may be due to a more efficient hepatic clearance in vivo. The inhibitory effect of dehydrocholate in vivo has been reported frequently (references see 54).

The nature of the inhibitory effects of bile salts is unclear. Competitive inhibition of bile salts and organic anions in the hepatic uptake process as reported by Delage (13) cannot be a general explanation, because our previous studies (53, 54) indicated, that also hepatic uptake of organic cations is inhibited.

The stimulatory effect of taurocholate on hepatic uptake of DBSP, recently reported by Marinovic (36), could not be confirmed in the present studies, nor in our previous studies with isolated hepatocytes (53).

In conclusion, this study indicates, that the effect of bile salts on hepatic transport of *DBSP* is dependent on

a. the dose of DBSP; especially with higher doses a stimulatory effect on maximal biliary excretion is found
b. the dose of bile salts; low doses have stimulatory effect on biliary excretion, high doses at the same time can inhibit hepatic uptake. This implies that the dosage regime of bile salts is important. Infusions of bile salts below maximal

hepatic removal rate will have stimulatory effects on biliary excretion, high infusion rate or bolus injections with high doses may cause concomittant inhibitory effects on hepatic uptake. Also the intrinsic hepatic clearance of the bile salts may play a role, bile salts with low clearance will more rapidly lead to inhibitory plasma concentrations

c. the choleretic potency of the bile salt; bile salt with a high choleretic action will have the most pronounced stimulatory effects on biliary excretion of DBSP

These factors, which generally vary among the many studies on this topic may explain the conflicting data and conclusions concerning interactions of bile salts and the hepatic transport of drugs.

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SUPPLEMENT VII

BILE SECRETION AND BILE COMPOSITION IN THE FREELY MOVING UNANESTHETIZED RAT. INFLUENCE OF FOOD INTAKE ON BILE FLOW

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SUMMARY

1. In freely moving, unanesthetized rats bile flow was measured continuously over the whole day - night cycle. Bile composition was analyzed and the influence of food intake on bile flow was investigated.
2. In both sexes a distinct circadian variation of bile production was observed. The mean night time level was 50 % higher than the day time level for female rats and 38 % for male rats. A sharp decrease in secretion rate was prominent when the light switched on and bile flow gradually increased in the afternoon.
3. The pattern of food intake was positively correlated with the pattern of bile secretion. During fasting only the general level of bile flow decreased, but the circadian variation persisted. Refeeding again increased the mean level of bile flow.
4. The chenodeoxycholate - cholate ratio in these permanent bile fistula rats was higher than in "acute" bile fistula rats and changed during the day - night cycle. It decreased from 1.01 at 05.00 h to a minimum of 0.41 at 15.00 h.
5. During the day - night cycle the sodium, potassium, calcium and cholesterol concentrations were rather constant. The total bile salt concentration was only slightly changed, suggesting that both the bile salt dependent fraction and the bile salt independent fraction were subject to about the same circadian variations.

INTRODUCTION

The mechanisms regulating bile fluid formation have not been

fully elucidated. It has been proposed that bile fluid is composed of three fractions. First a fraction called the bile salt dependent fraction, which may be the result of the osmotic activity of bile salts excreted in bile and secondly the bile salt independent fraction. The origin of the latter fraction is proposed to be due to sodium transport into the bile, controlled by the membrane bound sodium-potassium ATPase activity (Erlinger, Dhumeaux, Berthelot & Dumont, 1970). However, recently some objections have been raised against this theory (Balabaud, Kron & Gumucio, 1977; Meijer, Vonk & Weitering, 1978). Both of the above mentioned bile fractions are of canalicular origin. The third fraction is a ductular fraction, which is inducible by the hormone secretin in some species (O'Maille, Richards & Short, 1966), but not in the rat (Shaw & Heath, 1972). Therefore, it is suggested that this last fraction does not play an important role in the bile formation of rats.

So far, little is known about the nervous and hormonal regulation of the various bile fractions. Vagal control of bile flow has been suggested (Tanturi & Ivy, 1938; Pissidis, Nyhus & Bombeck, 1973), but also contradicted (Debray, De La Tour & Rozé, 1974; Pass & Heath, 1976). Some intestinal hormones like gastrin or cholecystokinin have a stimulatory effect on bile flow (Jones & Grossman, 1970). Under normal physiological circumstances these hormones are released after ingestion of food. Contradictory data are available concerning the relation between food intake and bile secretion. A stimulatory effect of food intake on bile production in dogs has been reported (Fritz & Brooks, 1963; Nahrwold & Grossman, 1967; Jones & Grossman, 1969), whereas others observed that this phenomenon did not exist in rats (Shaw & Heath, 1972). Species variation may be an important factor in the observed differences.

The present study was undertaken to study the influence of food intake and food deprivation on bile secretion in freely moving unanesthetized rats. Bile flow was measured continuously over the whole day - night cycle by means of a permanently implanted bile fistula and bile was not permitted to reach the intestinal tract. The influence of the recirculation of bile

salts, the trauma of the operation procedure and the stress of a restraining cage was eliminated by this procedure.

Bile flow was found to display a clear circadian variation. Bile collected in this way was analyzed, to investigate to what extent the different bile fractions mentioned above, contribute to the variations in bile production during the day - night cycle.

METHODS

Surgical technique (for a permanent bile fistula)

The application of a permanent bile fistula allows a continuous estimation of bile flow during the whole day - night cycle in freely moving, unanesthetized rats.

All surgical tools and cannula were sterilized with heat. Rats were anesthetized with diethylether. A median incision about 1.0 cm long was made on the top of the head and the skin was pulled aside. The membranes were removed from the bones of the skull by rubbing with dry cotton-wool. Three holes were made in the skull with a dental trephine, one in a frontal bone and one in each parietal boneplate. It was essential to stay at a safe distance from the blood sinuses situated directly under the skull. Three small screws were fitted into these holes, but tightened in such a way that about 0.5 mm remained between the skull and the head of each screw. A midline incision of about 2 cm was then made, one centimeter caudal of the processus xyphoïdeus. With a small foreceps the bile duct was held about one centimeter before the first bifurcation to the liver lobes. This part of the bile duct was free of pancreas tissue. A small incision was made in the bile duct with a sharp needle (23G) and with another foreceps the silicon cannula was inserted through this incision. This silicon cannula consisted of a long part of 15 cm (inner diameter 0.6 mm; outer diameter 1.2 mm) and a short part of 1.5 cm (inner diameter 0.5 mm; outer diameter 0.9 mm) which was inserted for about 2 mm into the long part. These two parts were glued to each other with silicon glue. The short part

was inserted into the bile duct up to a nodule of silicon which had been placed 5 mm from its tip. This nodule was then sutured to the bile duct. Another thickening with glue at a distance of 3 cm from the tip was used for fixing the cannula to the abdominal wall. The long part was then drawn with a long forceps under the skin to the skull incision. The silicon tubing was connected to a short bent piece of stainless steel tubing (outer diameter 0.9 mm). The latter was embedded in a layer of acrylic glue, about 3 mm thick, spread over a triangular area of the skull between the screws. In this way the cannula was attached to the skull. After that the skin was sutured around the glue. The rat was prophylactically injected with 60.000 i.u. penicillin. The total duration of this cannulation procedure was about one hour. After surgery, rats recovered rapidly from the operation and required no special care. Food intake returned to normal levels after a single day depending on the skilfulness of the surgeon. After surgery at least one week was allowed for recovery before the rats were used for the experiments (Light, Witmer & Vars, 1959). When no experiments were performed, a polythene tube was pushed over the stainless steel tubing. This tubing (6 cm) was carefully bent by heat, allowing the bile to flow on the wood-shavings.

The animals could be kept alive producing bile for several months and remained in good physical condition as could be seen in the regular feeding pattern and a normal increase in body weight. Some of the animals with a permanent fistula were sacrificed for electronmicroscopical and histochemical analysis of the liver. No abnormalities could be detected in appearance of ultrastructure of the hepatocytes by an electronmicroscopical analysis. Hepatic enzyme activity of alkaline phosphatase, 5'-nucleotidase, nucleoside polyphosphatase (ATPase), leucyl-ß-naphtyl amidase, acid phosphatase, ß-glucuronidase, glucose-6-phosphatase, non-specific esterase, succinate dehydrogenase, isocitrate dehydrogenase, ß-hydroxybutyric acid dehydrogenase, glucose-6-phosphate dehydrogenase and α-glycerophosphate were investigated by enzyme histochemical techniques. There was no significant difference in the activity and distribution of these

enzymes six weeks after the operation procedure between the animals with a permanent bile fistula and control animals.

Maintenance of animals and experimental procedure

Male and female rats were maintained in individual plexiglass chambers (25 x 25 x 30 cm) on wood shavings, at a room temperature of 20°C. A standard diet was presented by a rack attached to one of the walls of the cage. Lights were on from 07.00 h to 19.00 h.

During the experiments, a long polythene tubing of about 90 cm (inner diameter 0.75 mm, outer diameter 1.45 mm) was connected to the stainless steel tubing. The other end was attached to a fraction collector outside the cage at the same level as the cage floor. In order to prevent torsion of this long tubing a swivel joint designed by Strubbe (1974) was used. A thin thread for attaching a counter weight was tied to the polythene tube just above the swivel. The tubing was protected against gnawing with a stainless steel coil. A diagram of the experimental setup is shown in Fig. 1.

The occurrence of food intake during the whole day - night cycle was registered making use of a movable bar just in front of the food rack. When the animals were eating, the bar was pressed down, closing an electrical circuit which was recorded. To study the pattern of food consumption the following procedure was used. The twenty four hour period was divided into periods of five minutes. When eating was registered during this five minutes period a positive score was made. The mean number of positive scores was determined for each half hour of the day - night cycle over seven successive days.

Chemical analysis

Bile acids were determined according to the method of Roovers, Evrard & Vanderhaeghe (1968) and Van Berge Henegouwen, Ruben & Brandt (1974) with minor modifications. After methylation the bile acids were chromatographed as their trifluoroacetate derivatives on a column of 1 % OV-210 on Cromosorb W-HP

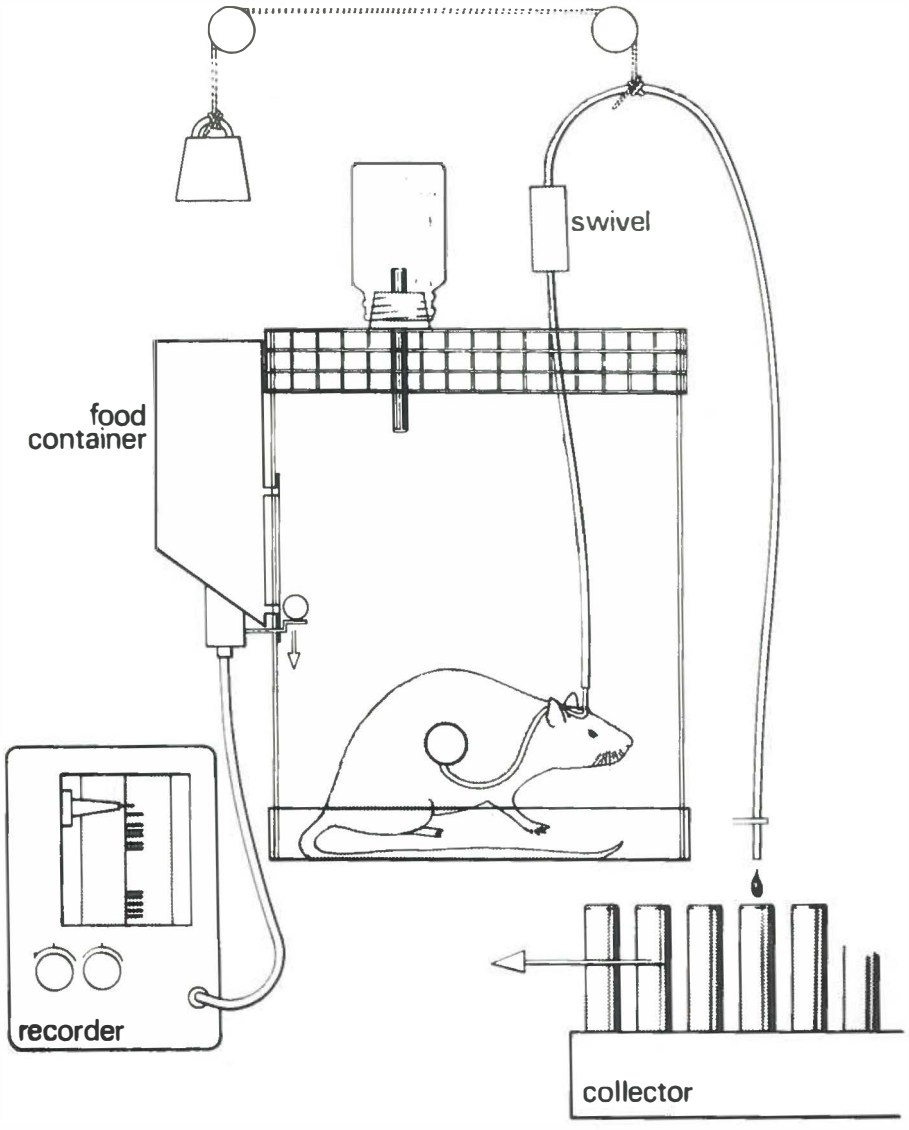

Fig. 1. A diagram of the experimental setup of a permanent implanted bile fistula in a freely moving rat, allowing continuous measuring of bile flow and food intake. (Design D. Visser).

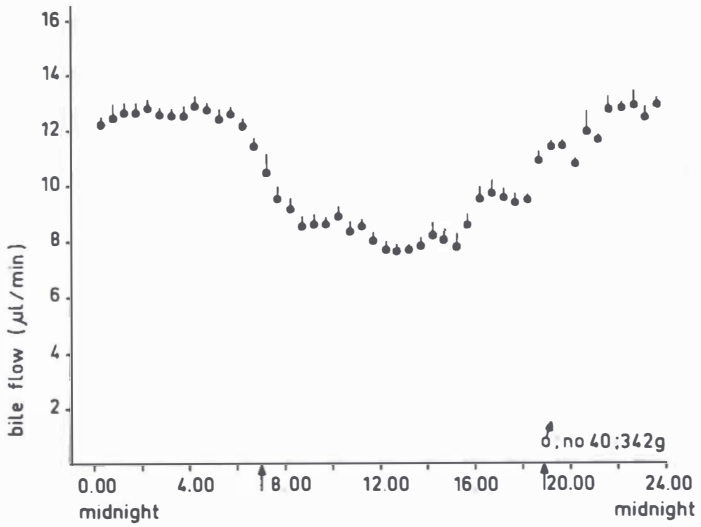

Fig. 2. Bile flow (µl/min) measured in one male rat over four days. Mean values ± SEM. ↑ = switch on/off of the lights.

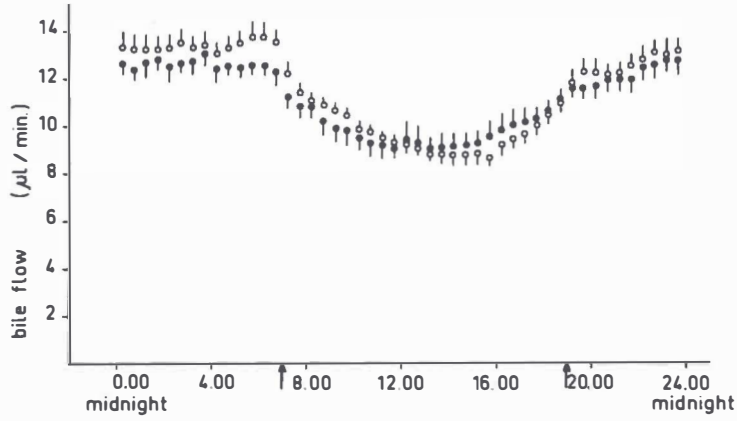

Fig. 3. Bile flow (µl/min) in male rats (●——●; mean body weight 335 ± 21 g) and female rats (○——○; mean body weight 239 ± 11 g) of the same age (six months). Mean values ± SEM of six animals; each animal measured for from three to six days.

100-120 mesh and analysed by flame ionization in a Packard-Becker Model 419 gaschromatograph.

Sodium and potassium were determined by flame spectrophotometry using a model 450 Corning-EEL flame photometer.

Calcium was determined by a complexometric titration with ethylene-glycolbis (aminoethylether)-tetra-acetic acid (EGTA). Detection of the end-point was obtained with calcein as fluorophore (Jackson, Breen & Chen, 1962).

Cholesterol was determined according to the method of Abell, Levy, Brodie & Kendall (1952).

Statistical analysis

Kendall's rank correlation test (Kendall, 1955) was used to evaluate whether a correlation existed between food intake and bile flow. The level of significance selected was $p < 0.05$.

RESULTS

Bile flow was measured continuously in the unanesthetized rats in fractions of thirty minutes. Fig. 2 illustrates a representative example of bile flow over the whole day - night cycle. This shows the mean value of one rat measured during four days. Bile flow was highest during the night and sharply decreased when the light switched on. From about 15.00 h a gradual increase in bile flow occurred. In general other rats displayed the same circadian patterns in bile secretion. The mean bile flow rate of six male rats and six female rats of about the same age (6 months) is plotted in figure 3. The circadian variations in bile flow could be observed in both sexes. The increase in bile flow between 15.00 and 20.00 h was more gradual in male rats than in female rats. The mean night time level of bile flow was 50 % higher than day time level for female rats and 38 % for male rats. Although the absolute amount of bile produced was about the same in both sexes (16.0 ml/day for males and 16.5 ml/day for females) female rats have a 44 % higher bile flow than males, when mean body weight is taken into consideration, which were 239 g and 335 g respectively.

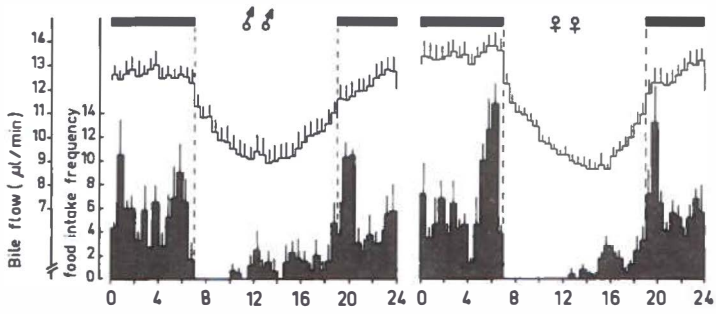

Fig. 4. Food intake pattern (black bars) and bile flow (——) in male and female rats.

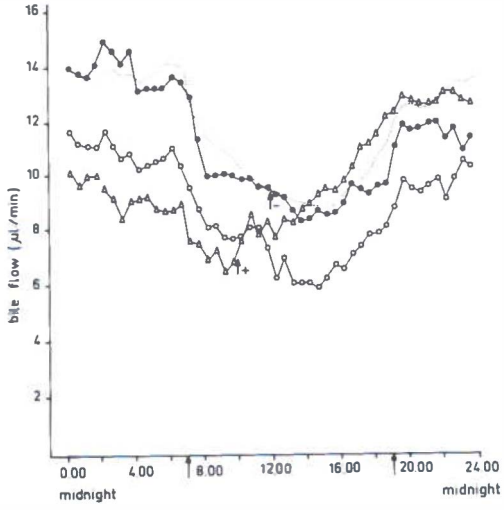

Fig. 5. Influence of fasting on bile flow in female rats. Mean values, n = 4. ----, normal bile flow in female rats; ●——●, bile flow 1° day; o——o, bile flow 2° day; Δ——Δ , bile flow 3° day. †- = start of food deprivation; †+ = restoration of food.

One of the factors influencing the circadian variation in bile flow may be food intake. Therefore, the patterns of food consumption were studied in male and female rats by automatic registration of food intake. Fig. 4 gives for each half hour, the mean frequency and standard error of food intake together with the mean bile flow in five female and four male rats. For both sexes a significant correlation ($p < 0.01; \tau = 0.48$ Kendall rank correlation) existed between the two parameters. This could mean that food intake might be responsible for the circadian variation of bile flow. However, the circadian rhythm persisted over 48 hours of fasting (Fig. 5), which contradicts this suggestion. A progressive decrease in mean flow level could be observed over a two days fast. Upon refeeding an immediate increase in bile flow was found.

As illustrated in figure 2, small irregularities are superimposed on the diurnal fluctuation in bile production. These were a reproducible characteristic for each individual. Food intake, which also showed a characteristic pattern for each

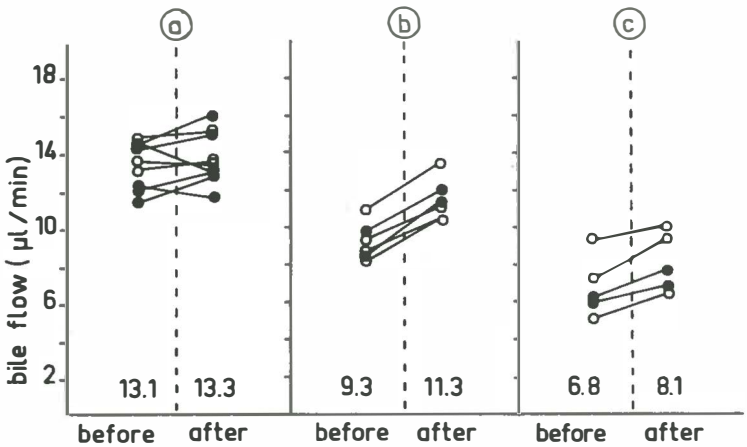

Fig. 6. Food consumption and bile flow (µl/min) in male rats (●——●) and female rats (o——o). a) Bile flow before and after the first meal after midnight (0.00 - 02.00 h). b) Bile flow before and after the first meal after noon (12.00 - 15.00 h). c) Bile flow before and after restoration of food after 48 h of fasting. "before" is the last period of 30 minutes *before* food consumption and "after" the first period of 30 minutes *after* food consumption. Mean values in each period are presented.

individual might be responsible for these small variations. To test this hypothesis the individual experiments were examined, in which bile production and food intake were measured for one day in one animal. Measurements of several animals were tested in such a way. Meal onset tended to be followed by a peak in bile flow, but this effect was not statistically significant over the whole day - night cycle. When the same statistical analysis was made for the correlation between bile flow and meals taken within a restricted period of the day or night, different results were obtained. For the first meal taken between 00.00 and 02.00 h again no statistically significant correlation was found (Fig.6a), but a significant positive correlation between stimulation of bile flow and food consumption was observed in the case of the first meal taken between 12.00 - 15.00 h (Fig. 6b). As the peak is always followed by a decrease in bile flow,it can not be caused by the circadian increase in bile flow alone. Fig. 6c illustrates the increase in bile flow after administration of food to rats which had fasted for 48 hours. A positive correlation between both parameters was also found for this case.

To determine the relative contribution of the bile salt dependent and bile salt independent fraction to changes in bile flow during the day - night cycle, the biliary concentration of some electrolytes, cholesterol and various bile salts were determined. If changes in only one of the bile fractions were responsible for the circadian variation, a change in, for instance, bile salt concentration would be expected. The total conjugated bile salt concentration in male rats was only slightly increased in the 18.00 - 24.00 h period (Fig. 7b). A comparison of the 12.00 - 14.00 h period with the 00.00 - 02.00 h period revealed a 38 % increase in bile flow, but only a 4 % increase in the total bile salt concentration.

The cholesterol concentration was rather constant over the day - night cycle (Fig. 7b). Fig. 7a illustrates, that the concentration of sodium, potassium and calcium also did not markedly change over the day - night cycle. This implies that the output of electrolytes in these animals is about 38 % higher in the night time than during day time. In single experiments

the concentration of calcium showed remarkable irregular fluctuations, the origin of which is not known.

The ratio of conjugated chenodeoxycholate to conjugated cholate clearly changed during the day - night cycle. This ratio reached a maximum of 1.01 at 06.00 h, decreased at the onset of light to a minimum of 0.41 and increased again when the lights switched off. The concentrations of unconjugated cholate and unconjugated chenodeoxycholate were only 0.5 mmoles/l and 0.2 mmoles/l respectively, which implies that about 95 % of the excreted bile salts were conjugated. Since the concentrations of both unconjugated bile salts as well as the concentrations lithocholate and deoxycholate were low (< 0.1 mmoles/l) no diurnal changes in the biliary concentration of these bile salts

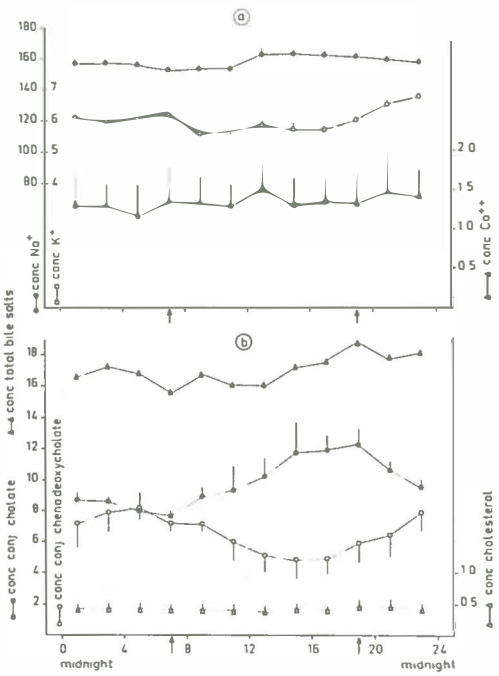

Fig. 7. Composition of bile of male rats during the day - night cycle measured one month after the operation a) concentration in mmoles/l of sodium (●——●), potassium (○——○) and calcium (▲——▲). Mean values ± SEM; n = 4. b) concentration in mmoles/l of conjugated cholate (●——●), conjugated chenodeoxycholate (○——○), total bile salts (▲——▲) and cholesterol (Δ——Δ). Mean values ± SEM; n = 3.

could be established. Comparison with bile from male, pentobarbital-anesthetized rats of the same strain (*acute* bile fistula rats) (Table I) showed that in acute bile fistula rats the conjugated chenodeoxycholate-conjugated cholate ratio is clearly different, the biliary concentration of conjugated cholate is lower, bile flow is much higher and total bile salt output is about the same as in the chronic bile fistula rats at noon (Vonk et al., unpublished work). The output of unconjugated bile salts is only slightly smaller (< 3 %) in acute bile fistula rats.

DISCUSSION

Circadian variations occur in a large number of physiological processes (Altman & Dittmer, 1973). In the liver of rats they have been shown for both cholesterol and/or bile salt synthetic activity (Back, Hamprecht & Lynen, 1969; Danielsson, 1972; Mitropoulus, Balasubramaniam, Gibbons & Reeves, 1972; Danielsson, 1973), as well as for sodium-potassium dependent ATPase and magnesium dependent ATPase (Bakkeren, 1968). Because bile flow is supposed to be regulated by bile salt output and ATPase

	permanent bile fistula rats		acute bile fistula rats
	12.00 - 14.00	00.00 - 02.00	
bile flow (µl/min)	9.1 ± 0.7	12.6 ± 0.5	18.3 ± 0.6
conc.conj.cholate (mM)	10.2 ± 1.2	8.7 ± 0.5	7.2 ± 0.5
conc.conj.chenodeoxycholate (mM)	5.1 ± 1.1	7.2 ± 1.6	1.1 ± 0.1
conc.total bile salt (mM)	16.0 ± 1.8	16.6 ± 1.2	9.0 ± 0.6
output total bile salt (nmoles/min)	146 ± 20	210 ± 13	165 ± 16

Table I. Bile flow, bile salt concentrations and bile salt excretion in permanent bile fistula rats at noon (12.00 - 14.00h) and at midnight (00.00 - 02.00h) and in rats with an "acute" bile fistula. In the latter experiments bile was collected from male, pentobarbital anesthetized animals at noon, about 1.5 h after the operation procedure.

activity (Erlinger & Dhumeaux, 1974), a circadian variation in bile flow would also be expected. Recently Ho & Drummond (1975) and Balabaud, Noël, Béraud & Dangoumau (1975) demonstrated such a circadian rhythm in bile flow using rats in restraining conditions and pentobarbital anesthetized rats respectively. The present study with free-moving unanesthetized rats confirmsthese results. The technique used in these studies allows a frequent sampling of bile over long periods which permits the study of acute fluctuations in bile flow. A sharp decrease in bile flow could be observed when the lights switched on, while the increase at the end of the day was more gradual.

One of the factors responsible for changing the rate of bile secretion may be food intake (Fritz & Brooks, 1963; Nahrwold & Grossman, 1967; Jones & Grossman, 1969). Therefore, we measured bile flow and food uptake simultaneously. The food intake behaviour of the rats was similar to that described by Siegel (1961) and Le Magnen & Tallon (1966). A positive correlation between food intake and bile flow within the same thirty minute interval was observed. In order to see whether this correlation represented a causal relationship, a comparison was made with the fasting experiments. During fasting the circadian variation persisted, although the general level of bile flow was decreased. This indicates that food intake is at least partly responsible for the general level of bile flow, but not for the day - night fluctuations. Similar relations were found for the activity of some digestive enzymes in the small intestine of the rat (Saito, Murakami, Nishida, Fujisawa & Suda, 1976). When the relationship between small variations in bile flow and food consumption was investigated under different conditions, a positive correlation between these parameters was found to exist at refeeding after 48 h of fasfting, at noon, but not during the night, when bile flow if high. The mechanism which governs the food-dependent and food-independent activation of bile flow is not known. With regard to the food-dependent activity it has been suggested that the presence of food in the intestinal tract releases hormones like cholecystokinin, which has choleretic properties (Jones &

Grossman, 1970). Increased secretion of this hormone might, therefore, be responsible for the food-dependent bile secretion, but alternatives deserve to be considered. The food-independent regulation might be controlled jointly by many factors (Ho & Drummond, 1975). Conflicting evidence exists concerning one of these factors, the vagus nerve (Tantury & Ivy, 1938; Pissidus et al., 1973; Debray et al., 1974).

It would be of interest to know whether the bile salt dependent fraction and bile salt independent fractionsof bile are subject to the same circadian variation. The total bile salt concentration in the period from 12.00 - 14.00 h was 16.0 mmoles/l (Table I). Assume that *only* the bile salt dependent fraction is subject to a circadian variation and at noon this fraction is 50 % of the total bile flow (Erlinger & Dhumeaux, 1974). In the period of 00.00 - 02.00 h which has an increased bile flow of 38 % the total bile salt concentration would be expected to be 20.4 mmoles/l (when the bile salt dependent fraction of the total bile flow is less than 50 %, this concentration becomes higher). Because the observed total bile salt concentration in the period from 00.00 - 02.00 h is only 16.6 mmoles/l, it can therefore be concluded that the bile salt independent fraction is also subject to circadian variation. This contradicts Balabaud et al. (1975), who observed that the bile salt concentration in bile was decreased during night time and concluded that the circadian rhythm in bile flow in pentobarbital anesthetized rats was due to fluctuations in the bile salt independent fraction.

A change in the chenodeoxycholate-cholate ratio over the day - night cycle was observed in our experiments. The normal synthesis of both bile acids (Danielsson, 1973) includes the synthesis of cholesterol from acetyl CoA with the rate limiting enzyme hydroxymethylglutaryl-SCoA and subsequently a conversion to 7 α-hydroxy-cholest-4-en-3-one with the rate limiting enzyme cholesterol 7 α-hydroxylase. This intermediate 7 α-hydroxy-cholest-4-en-3-one can be metabolized both to chenodeoxycholate as well as to cholate. The first step in the biotransformation of the last compound is regulated by the 12 α-hydroxylation of

7 α-hydroxy-cholest-4-en-3-one. The activity of this last enzyme alone (Cohen, Raicht, Nicolau & Mosbach, 1975) or the ratio of the activities of the microsomal 26-hydroxylase and the 12 α-hydroxylase (Björkhem, Danielsson, Gustafsson, 1973) may play a major role in determining the ratio between cholate and chenodeoxycholate formed from cholesterol. The reason for changes in the chenodeoxycholate-cholate ratio is not clear. Perhaps not all enzyme-activities in the bile salt synthesis pathway show the same diurnal variation as hydroxymethylglutaryl-SCoA and 7 α-hydroxylase of cholesterol (Danielsson, 1972; Mitropoulus et al., 1972). Circadian variations in the ratio of chenodeoxycholate-cholate may be explained by a deviating circadian fluctuation of 12 α-hydroxylase activity. However, Danielsson (1972) concluded from experiments in vitro that 12 α-hydroxylation of 7 α-hydroxy-cholest-4-en-3-one did not show circadian variations in enzyme activity, in contrast to 7 α-hydroxylation of cholesterol (Mitropoulos, Balasubramaniam & Myant, 1973).

The 12 α-hydroxylase activity or the 12 α-hydroxylase/26 α-hydroxylase activity ratio may also be responsible for the higher ratio of chenodeoxycholate-cholate in isolated hepatocytes (Anwer, Kroker & Hegner, 1975) and in the chronic bile fistula rats used in our experiments. In vitro experiments (Johansson, 1970; Björkhem & Danielsson, 1974), however, contradict this suggestion. Our bile fistula rats, which do not possess an enterohepatic circulation, may have a derepressed synthesis of bile salts. In these animals the synthesis of bile salts is 272 μmoles/day, while Eriksson (1957) reported a value of about 112 μmoles/day and Ho and Drummond (1975) 422 μmoles/day. For rats with intact enterohepatic circulation this value amounts to about 13 μmoles/day. This derepression of the bile salt synthesis may be higher for the enzyme hydroxymethylglutaryl-SCoA reductase and 7 α-hydroxylase of cholesterol than for 12 α-hydroxylase of 7 α-hydroxy-cholest-4-en-3-one, which also explains a higher relative amount of chenodeoxycholate.

This study with unanesthetized, freely moving rats indicates not only the existence of a diurnal variation in bile flow, but

also in biliary bile salt composition. The influence of these variations on hepatic functions, like the biliary transport process, is the subject of further study.

ACKNOWLEDGEMENTS

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SUPPLEMENT VIII

BILIARY EXCRETION OF DIBROMOSULPHTHALEIN IN THE FREELY MOVING UNANESTHETIZED RAT. CIRCADIAN VARIATION, EFFECT OF FASTING AND PENTOBARBITAL ANESTHESIA

R.J. VONK, E. SCHOLTENS and J.H. STRUBBE

SUMMARY

1. In unanesthetized, freely moving rats, which displayed a circadian rhythm in bile flow, hepatic transport of dibromosulphthalein was investigated at midnight, when bile flow was high and at noon when bile flow was lower. The influence of pentobarbital anesthesia and fasting on hepatic transport of dibromosulphthalein was also studied. The data obtained with these permanent bile fistula rats with a complete interrupted enterohepatic circulation of bile were compared with the data of rats with an intact or shortly interrupted enterohepatic circulation of bile, to evaluate the influence of the enterohepatic circulation of bile (salts) on the hepatic transport process.

2. Maximal biliary transport of dibromosulphthalein was subject to circadian variations: the biliary transport maximum at night was 25 % higher than at noon, while maximal biliary concentration was not significantly altered. The distribution volume was increased with 21 % during nighttime, but the primary hepatic clearance constant was not changed.

3. Pentobarbital anesthesia decreased the maximal biliary concentration and the maximal biliary excretion rate of dibromosulphthalein, but the primary hepatic clearance constant was not changed.

4. 48 hours of fasting changed the primary hepatic clearance constant as well as the biliary excretion of DBSP. The maximal biliary excretion was diminished, while maximal biliary concentration was increased.

5. Interruption of the enterohepatic circulation of bile did not

change the primary hepatic clearance constant of dibromosulphthalein, but presumably decreased biliary excretion of the drug. 6. This study clearly indicates, that time of the day, feeding conditions, the use of anesthetics and interruption of the enterohepatic circulation of bile are important determinants in biliary excretion of cholephilic dyes.

Abbreviations: DBSP, phenol 3,6-dibromphthalein disulfonate (dibromosulphthalein); Tm, transport maximum of biliary excretion (of DBSP); Cm, maximal biliary concentration (of DBSP); $t_{\frac{1}{2}}$, half life time; V_c, apparent volume of the central compartment; r, rate constant; K_{12}, primary hepatic clearance constant (see Methods).

INTRODUCTION

Circadian variations occur in a large number of processes in the living organism (Altman & Dittmer, 1973). Concerning the bile, two factors which are suggested to be involved in its formation, are subject to circadian variations. Back, Hamprecht & Lynen (1969), Danielsson (1972; 1973) and Mitropoulus, Balasubramaniam, Gibbons & Reeves (1972) showed a diurnal variation in hepatic cholesterol and/or bile salt synthetic activity and Bakkeren (1968) reported the same concerning hepatic ATPase activity. Bile flow and bile salt output also vary during the day - night cycle (Mitropoulus, Balasubramaniam & Myrant, 1973; Ho & Drummond, 1975; Vonk, Van Doorn & Strubbe, 1978[c]). Food intake proved not to be responsible for the fluctuation in bile flow, but food deprivation decreased the overall bile flow level (Vonk et al., 1978[c]). As biliary excretion rate of some endogenous and exogenous compounds is dependent on bile flow or bile salt excretion (Boyer, Scheig & Klatskin, 1970; Goresky, Haddad, Kluger, Nadeau & Bach, 1974; Vonk, Jekel & Meijer, 1975), Tm of these compounds is expected to change during the day - night cycle and under different feeding conditions. The present study aims to investigate this. The hepatic transport of dibromosulphthalein (DBSP) was investigated

at midnight when bile flow is high and at noon when bile flow is low and further after a fasting period of 48 hours. The compound DBSP was chosen, because it is not metabolized by the liver (Javitt, 1964; Klaassen & Plaa, 1968); it has no distinct toxic effects and its biliary excretion can be stimulated by administration of choleretics (Vonk et al., 1975; Mahu, Duvaldestin, Dhumeaux & Berthelot, 1977).

In the experiments unanesthetized freely moving rats were used, provided with a permanently implanted bile fistula and heart catheter, allowing continuous bile collection and frequent sampling of blood. In these animals bile was not permitted to reach the intestinal tract, which eliminates possible influences of recirculating bile salts on hepatic uptake of drugs (Marinovic, Glasinovic, Semelle, Boivieux & Erlinger, 1977; Vonk, Jekel, Meijer & Hardonk, 1978[a]). Each animal served as its own control. The pharmacokinetics of DBSP in these animals with a permanently interrupted enterohepatic circulation of bile salts were compared with those in animals with a shortly interrupted enterohepatic circulation and animals with an intact enterohepatic circulation, in order to evaluate the influence of bile salts originating from the enterohepatic circulation on the hepatic transport process.

Generally pharmacokinetic studies in animals are performed during anesthesia. Because anesthetics may influence pharmacokinetic parameters, in this study also the effect of pentobarbital (Nembutal[R]) on hepatic transport of DBSP was investigated.

METHODS AND MATERIALS

Chemicals

DBSP was obtained from Société d'Etudes et de Recherches Biologiques (SERB), Paris, France, and pentobarbital (Nembutal[R]) from S.A. Abbott, France.

Maintenance of animals

Male rats of about 340 g were maintained in individual plexiglass cages (25 x 25 x 30 cm) on wood shavings, at a room

temperature of 20°C. A standard diet was supplied ad libitum (except in the deprivation experiments) by a food container attached to one of the walls of the cage. Water was freely available at all times. Lights were on from 07.00 h to 19.00 h.

Surgical technique

"Permanent bile fistula rats" were provided with permanently implanted bile fistulae as described extensively elsewhere (Vonk et al., 1978[C]). The method allows continuous sampling of bile during the whole day - night cycle. Moreover, these rats were provided with a permanent heart catheter via the jugular vein in the right atrium as described by Steffens (1969). Using this technique it is possible to inject substances directly in the general circulation and to sample blood. Thus, plasma disappearance and biliary excretion of substances can be measured in undisturbed, unanesthetized and freely moving rats. After surgery at least one week was allowed before the rats were used for the experiments, to establish a new level of bile flow and bile salt synthesis (Light, Witmer & Vars, 1959).

"Non bile fistula rats" were only provided with a permanent heart catheter as described above.

"Acute bile fistula rats" are rats which had an interrupted enterohepatic circulation of bile salts for about one hour before the drug was injected and which were pentobarbital anesthetized. The surgical technique used for these animals was described earlier (Vonk et al., 1975).

Experimental procedures

In experiments with permanent bile fistula rats one end of a polythene tube of about 90 cm (inner diameter 0.75 mm) was connected to the stainless steel tubing at the head of the animal. The other end was attached outside the cage at the same level as the cage floor to a fraction collector. Bile was collected in fractions of ten minutes. The dead space in the tubing outside the bile duct, which was constant and amounted to 0.50 ml, was

corrected for in all experiments. In order to prevent torsion of the tube connected to the animal, a swivel joint as described by Strubbe (1974) was used. A thin thread for attaching a counter balancing weight was tied to the polythene tube just above the swivel. Further the tubing was protected against gnawing with a stainless steel coil. DBSP was injected intracardially by a single dose of 150 µmoles/kg, which allows measurement of plasma concentrations and achieves biliary excretion rates at Tm values (Vonk et al., 1975). Blood samples of 0.25 ml were taken in heparinized tubes. When experiments in the night were performed, dimmed lights were used in order not to disturb the circadian rhythm. The dosage of DBSP in the night was based on body weight at daytime. During pentobarbital experiments the temperature of the animals was kept between 37.5 - 38.0°C by a heating lamp to prevent hypothermia, which influences the biliary excretion process (Roberts, Klaassen & Plaa, 1967). During fasting besides tap water, saline (0.9 % w/v NaCl) was available to the animals. The dosage of DBSP after 48 h of fasting was based on body weight before the fasting period. In all experiments with permanent bile fistula rats, unless specified otherwise, we determined plasma concentration of DBSP at t = 1, 2, 3, 4, 8, 12, 16 and 28 min, Cm, Tm and percentage of recovery after 4 h of DBSP.

In non bile fistula rats plasma concentrations of DBSP were determined at the same time intervals as described above.

In acute bile fistula rats plasma concentrations of DBSP, Cm and Tm were determined.

Chemical analysis

DBSP was determined in bile and plasma. 50 µl plasma was diluted with 5 ml 0.1 N NaOH and measured spectrophotometrically at 580 nm. 10 µl bile was diluted with 10 or 20 ml 0.1 N NaOH and measured in the same way.

After injection of DBSP, bile samples were subjected to thin layer chromatography in order to study biochemical modifications. Thin layer chromatography with Silicagel 60 F (Merck A.G.) and n-butanol, acetic acid and distilled water (4 : 1 : 2) indicated

that DBSP was not metabolized, which is in accordance with Javitt (1964) and Klaassen & Plaa (1968). We did not find any evidence for glucuronidation of DBSP in our animals, which has been shown to occur in Gunn rats (Javitt, 1976).

Pharmacokinetic analysis

The plasma disappearance curves of DBSP were analyzed according to a two compartment model with elimination from the peripheral compartment as described by Richards, Tindall & Young (1959). Elimination from the peripheral compartment was concluded, because the assumption of elimination from the central compartment delivered conflicting evidence concerning the biliary excretion data. The rate constant of hepatic uptake (r_{12}) can be calculated from the equation $\frac{A\alpha + B\beta}{A+B}$; the rate constant of biliary excretion (r_{23}) as $a\beta/r_{12}$ and the rate constant of reflux from liver to plasma (r_{21}) as $\alpha + \beta - r_{12} - r_{23}$; α and β represent the slopes of the fast and the slow phase of the semi-logarithmically plotted plasma disappearance curve (Fig. 2) and A and B the intercepts of the Y axis after extrapolation of the α and β phase respectively. V_c was calculated as D(ose)/A + B) and expressed as percentage of body wt. The initial hepatic clearance constant (K_{12}) was calculated as r_{12} x (V_c x body wt./ 100).

The second part of the plasma disappearance curve of DBSP in this study was regarded as one monoexponential phase in the measured time course. The slope of this curve was dependent on the administered dose of DBSP (unpublished work). Therefore r_{23} was dependent on the dose, and the calculated values of r_{23} in this study are *relative* values; in contrast r_{12} and r_{21} were independent of the dose.

Statistical analysis

Statistical significance of differences between the various groups of experiments was tested by the Wilcoxon test.

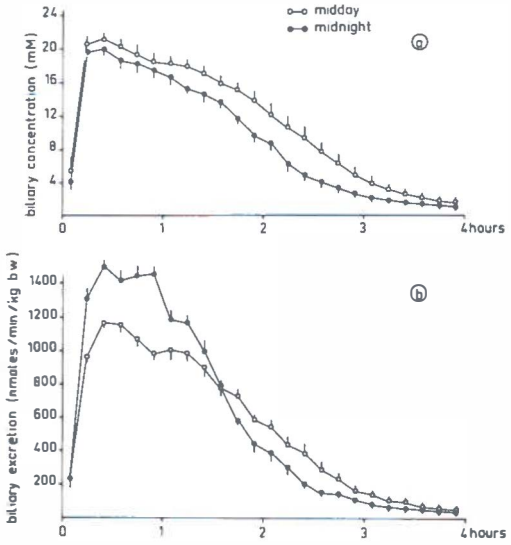

Fig. 1. Biliary excretion of DBSP in unanesthetized rats at noon (o——o) and at midnight (●——●) after injection of DBSP (150 µmoles/kg body weight).
a) biliary concentration in mmoles/l; b) biliary excretion rate in nmoles/min/kg body weight. Mean values of five animals ± SEM.

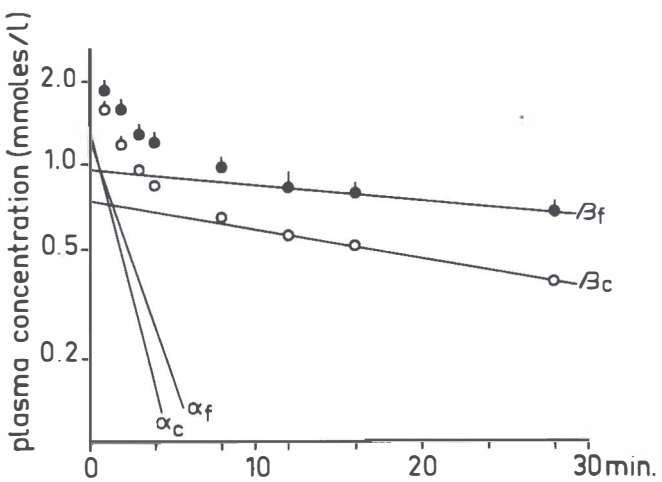

Fig. 2. Plasma disappearance of DBSP after injection of 150 µmoles/kg body weight at noon, in rats which had free access to food and water (o——o) (n = 5), and at noon in fasting rats (●——●) (n = 4). Mean values ± SEM.

RESULTS

Influence of time of the day

In previous studies (Vonk et al., 1978[c]) in unanesthetized permanent bile fistula rats,bile flow and bile salt output were measured and both parameters were compared in the period of 00.00 - 02.00 h and 12.00 - 14.00 h. Bile flow was increased by 38 % during nighttime, while total bile salt concentration was not significantly different in both periods.

In the present study DBSP was injected in male rats in a dose of 150 µmoles/kg body weight at two different times of the day - night cycle, 00.00 h and 12.00 h. Biliary DBSP concentration (Fig. 1a) and biliary excretion rate of DBSP (Fig. 1b) were compared in both situations. Biliary concentration during nighttime tended to be lower than during daytime, but this effect was not statistically significant during maximal excretion rates with biliary concentrations of 19.5 and 20.3 µmoles/l respectively (Table I). Tm during nighttime was significantly increased by about 30 %. Bile flow during nighttime at maximal DBSP excretion was increased with 39 %; the same change in bile flow was observed in control studies without DBSP. The total recovery of DBSP in bile after four hours was not significantly different at noon or at midnight (Table I).

In Fig. 2 the plasma disappearance of DBSP is plotted semilogarithmically versus time. The plasma disappearance curve was composed of at least two phases, a slow (β) phase with $t_{\frac{1}{2}}$ (β) of 27.4 min and a fast (α) phase with $t_{\frac{1}{2}}$ (α) of 1.2 min (corrected for the slow phase). No significant difference could be observed in both $t_{\frac{1}{2}}$'s at noon or midnight (Table I). A 20 % increase of V_c during nighttime was found. The rate constant of hepatic uptake (r_{12}) was decreased during nighttime, while the rate constant of biliary excretion (r_{23}) was increased. The rate constant for reflux of DBSP from liver to plasma (r_{12}) was not statistically significant altered.

TABLE I

	Permanent bile fistula rats				acute bile fistula rats	non bile fistula rats	
	noon (5)	midnight (5)	pentobarbital (6)	fasting (4)	pentobarbital (6)	noon (4)	pentobarbital (4)
C_m mmoles/l	20.3 ± 0.7	19.5 ± 0.8	17.4 ± 0.8 ‡	23.9 ± 1.7*	22.4 ± 0.6**		
T_m nmoles/min/kg/b.w.	1180 ± 30	1480 ± 50**	1040 ± 40*	780 ± 70**	1670 ± 40**		
bile flow µl/min	18.3 ± 0.9	23.9 ± 0.6*	17.8 ± 1.0	11.0 ± 0.7**	22.8 ± 0.9**		
recovery (4 h) %	90 ± 2	93 ± 3	83 ± 1*	81 ± 4*	> 85		
$t_{\frac{1}{2}}$ (α) min	1.2 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.7 ± 0.1**	1.0 ± 0.0**	1.6 ± 0.1**	1.4 ± 0.2
$t_{\frac{1}{2}}$ (β) min	27.4 ± 1.4	24.3 ± 2.7	39.7 ± 2.1**	46.7 ± 3.7**	26.1 ± 2.0**	19.8 ± 1.1**	23.7 ± 0.4*
V_c % $^v/_w$	6.9 ± 0.3	8.3 ± 0.2**	6.9 ± 0.3	6.5 ± 0.3	4.7 ± 0.3**	6.3 ± 0.2	6.6 ± 0.6
r_{12} min^{-1}	0.388 ± 0.020	0.282 ± 0.040*	0.355 ± 0.022	0.245 ± 0.018**	0.507 ± 0.026**	0.265 ± 0.014**	0.284 ± 0.013
r_{23} min^{-1}	0.039 ± 0.002	0.055 ± 0.007 ‡	0.027 ± 0.002**	0.026 ± 0.002**	0.039 ± 0.003**	0.067 ± 0.008**	0.044 ± 0.002**
r_{21} min^{-1}	0.182 ± 0.012	0.205 ± 0.028	0.169 ± 0.009	0.163 ± 0.009	0.212 ± 0.007*	0.149 ± 0.007	0.153 ± 0.011
K_{12} ml/min	8.32 ± 0.60	7.71 ± 1.02	7.78 ± 0.75	5.28 ± 0.30**	6.84 ± 0.23	7.19 ± 0.28	7.86 ± 1.10

Table I. Data concerning hepatic transport of DBSP in male rats in *permanent bile fistula rats* at noon, at midnight, during pentobarbital anesthesia at noon and during fasting at noon, in *acute bile fistula rats* during pentobarbital anesthesia and in *non bile fistula rats* at noon and during pentobarbital anesthesia at noon. Permanent bile fistula rats have a permanently interrupted enterohepatic circulation; in acute bile fistula rats enterohepatic circulation was interrupted for about one hour and non bile fistula rats have an intact enterohepatic circulation. The latter animals were only provided with a heart catheter. T_m is the maximal biliary excretion rate of DBSP calculated as the mean value of three consecutive periods of ten minutes with maximal biliary excretion. C_m is the maximal biliary concentration calculated as the mean value of three consecutive periods of ten minutes with maximal biliary concentration. Bile flow is calculated as the mean value of bile flow in the same periods. Recovery in bile after 4 h is given as the percentage of the dose. $t_{\frac{1}{2}}$ (α) and $t_{\frac{1}{2}}$ (β) are respectively the half lives of the rapid and slow phase of the plasma disappearance. V_c is the distribution volume of the central compartment, expressed as percentage of the body weight. r_{12} is the rate constant of primary hepatic uptake, r_{23} the rate constant of biliary excretion, r_{21} the rate constant of reflux from liver to plasma and K_{12} the primary hepatic clearance constant.
During fasting and at midnight the body weight at noon is used for calculating the dose, T_m and V_c. The values of the permanent bile fistula rats at midnight, during pentobarbital anesthesia and during fasting were compared with the same values of the permanent bile fistula rats at noon; the values of acute bile fistula rats during pentobarbital anesthesia were compared with those of permanent bile fistula rats during pentobarbital anesthesia. The values of the non bile fistula rats at noon were compared with the values of permanent bile fistula rats at noon; the values of non bile fistula rats during pentobarbital anesthesia were compared with those from non bile fistula rats at noon. $\dagger$: $p < 0.1$; * : $p < 0.05$; ** : $p < 0.01$; n is represented between parenthesis at the heading of each column.

Influence of pentobarbital anesthesia

The influence of pentobarbital anesthesia was investigated in two groups of rats: the permanent bile fistula rats and the non bile fistula rats. Pentobarbital anesthesia was performed at noon after intraperitoneal injection of 60 mg/kg body weight of Nembutal^{R}. The effect of pentobarbital was measured using the same animals of both groups without pentobarbital anesthesia as control (see Table I, column "noon" and "pentobarbital" of both groups of animals.

In permanent bile fistula rats maximal biliary concentration of DBSP decreased from 20.3 to 17.4 µmoles/l by pentobarbital anesthesia, while bile flow at C_m was not significantly different, which resulted in a decreased Tm (Table I). During pentobarbital anesthesia Tm was reached after about 50 min, while in the control situation this maximum was found after about 25 min (Fig. 3).

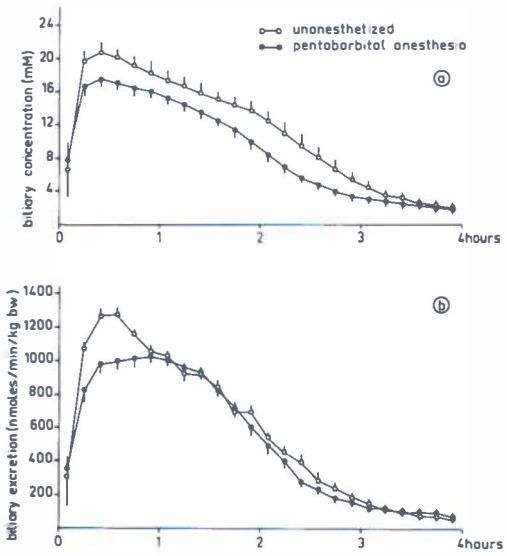

Fig. 3. Biliary excretion of DBSP in unanesthetized rats (o——o) (n = 5) and pentobarbital anesthetized rats (●——●) (n = 6) after injection of DBSP (150 µmoles/kg body weight) a) biliary concentration in mmoles/l; b) biliary excretion rate in nmoles/min/kg body weight. Mean values ± SEM.

Plasma disappearance of DBSP in permanent bile fistula rats during pentobarbital was retarded; $t_{\frac{1}{2}}$ (β) was prolonged from 27.4 to 39.7 min, while $t_{\frac{1}{2}}$ (α) was not much changed (Table I). r_{12} and K_{12} were not significantly changed, but r_{23} was decreased during pentobarbital anesthesia.

In non bile fistula rats the same effect of pentobarbital anesthesia was observed. r_{23} was decreased, while r_{12}, K_{12} and r_{21} were not significantly changed.

Influence of fasting

The influence of fasting was investigated in the permanent bile fistula rats (Fig. 4). After 48 hours of fasting most pharmacokinetic parameters were changed compared with control

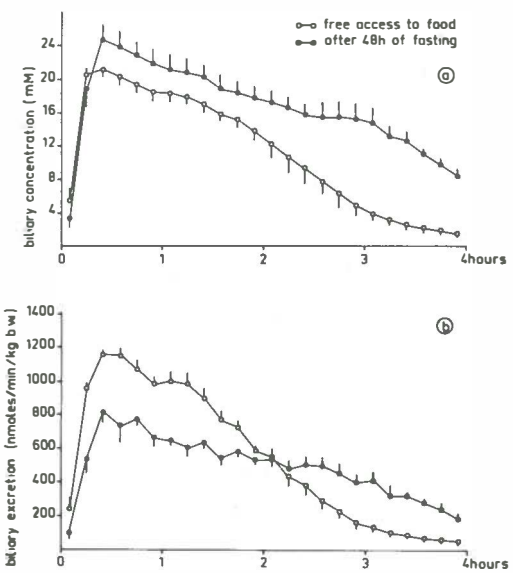

Fig. 4. Biliary excretion of DBSP in unanesthetized rats, which had free access to food and water (o——o) (n = 5) and in animals after 48 h of fasting (●——●) (n = 4) after injection of DBSP (150 µmoles/kg body weight). a) biliary concentration in mmoles/l; b) biliary excretion rate in nmoles/min/kg body weight. Mean values ± SEM.

values. (See table I, column "noon" and "fasting"). An increase in biliary concentration from 20.3 to 23.9 μmoles/l was found. Also other parameters of the biliary excretion process were changed: a diminished Tm with 34 % bile flow with 40 % and total recovery after 4 h with 10 %. Plasma disappearance was also retarded (Fig. 2); $t_{\frac{1}{2}}$ (α) and $t_{\frac{1}{2}}$ (β) increased and r_{12}, K_{12} and r_{23} decreased.

Comparison with acute bile fistula rats

To investigate the possible effects of the enterohepatic circulation of bile (salts), the pharmacokinetics of hepatic transport of DBSP in pentobarbital anesthetized rats with a permanent interrupted enterohepatic circulation of bile, were compared with the corresponding values in pentobarbital anesthetized rats with a shortly interrupted enterohepatic circulation of bile. (See table I, column "pentobarbital" of permanent and acute bile fistula rats). In acute bile fistula rats with a shortly interrupted enterohepatic circulation Cm, Tm and bile flow were 37 %, 73 % and 30 % higher respectively (Table I; Vonk et al., unpublished work). Also plasma disappearance was changed; $t_{\frac{1}{2}}$ (α) and $t_{\frac{1}{2}}$ (β) were diminished, r_{12}, r_{23} and r_{21} were increased. In these animals, in which another surgical procedure was used, the distribution volume was smaller. The K_{12}, however, was not significantly changed.

Comparison with non bile fistula rats

To investigate both the acute and chronic effect of recirculating bile salts on hepatic transport of DBSP, plasma disappearance of DBSP was measured in non bile fistula rats with and without pentobarbital anesthesia. The kinetics of plasma disappearance were compared with those in permanent bile fistula rats with and without pentobarbital anesthesia respectively. (Table I). In the unanesthetized non bile fistula rats both $t_{\frac{1}{2}}$'s, r_{12} and r_{23} were increased compared with the unanesthetized permanent bile fistula rats, but the K_{12} was not significantly

changed. In the anesthetized non bile fistula rats r_{12} was lower, r_{23} higher but K_{12} the same compared with anesthetized permanent bile fistula rats. r_{23} in non bile fistula rats was 66 % higher in the unanesthetized and 43 % higher in anesthetized animals compared with unanesthetized and anesthetized permanent bile fistula rats respectively.

DISCUSSION

Many factors may affect the hepatic uptake and biliary excretion of drugs. Hepatic blood flow influence hepatic uptake of drugs with high intrinsic hepatic clearance (Branch, Shand, Wilkinson & Nies, 1974). Binding of drugs to albumin may change hepatic uptake (Baker & Bradley, 1968; Meijer, Vonk, Keulemans & Weitering, 1977). Microsomal enzyme inducers like phenobarbital enhance biliary excretion of drugs (Fujimoto, Eich & Nichols, 1965). Co-medication may change hepatic transport of drugs in a competitive or non-competitive way. Moreover, changes in bile flow or bile salt output by administration of bile salts may stimulate (O'Maille, Richards & Short, 1966; Vonk et al., 1975) or inhibit (Bloomer, Boyer & Klatskin, 1973) the biliary excretion of drugs into bile. This study indicates that the hepatic transport of drugs is also subject to circadian variations. Tm of DBSP at nighttime is 25 % higher than Tm during daytime. Bile flow and total bile salt output in the permanent bile fistula rats is respectively 38 % and 44 % higher at nighttime than the corresponding values during daytime (Vonk et al., 1978[c]). It is uncertain, whether the increased biliary DBSP excretion is caused by an increased bile flow and/or bile salt output or is directly regulated by a so far unknown circadian factor. The observations that biliary excretion of DBSP can be enhanced by administration of choleretics like taurocholate or dehydrocholate (Vonk et al., 1975; Mahu et al., 1977) and the fact that maximal biliary concentration of DBSP is not significantly different at noon or at night favours the suggestion that bile flow and/or bile salt output regulate the biliary transport maximum of DBSP.

When plasma disappearance of DBSP is analyzed under these

conditions of unequal biliary output of DBSP, it was found that r_{23} was increased at night, but r_{12} decreased. Since at a given clearance the rate constant is inversely related to the apparent volume of the central compartment, (V_c) changes in the rate constant should be regarded in connection with the different values of V_c. This revealed that the initial clearance constant was not statistically significant changed, because of the increase V_c at nighttime. The increase in V_c may be caused by an increased plasma volume at night, when the animals have a higher activity and by consequence a well perfused vascular system.

Most studies concerning hepatic transport of drugs were performed with anesthetized animals. However, the trauma of the operation procedure may affect the excretion function (Light et al., 1959; Herman, Redinger, Small & Egdahl, 1971) and anesthetics may alter either directly or by an influence upon the bile production (Cooper, Eakins & Slater, 1976) the hepatic transport process. With our experimental setup we were able to study the influence of anesthetics on the hepatic transport process in unrestrained animals. We were especially interested in the influence of pentobarbital, because in most of our previous studies pentobarbital was used as anesthetic agent (Vonk et al., 1975; Meijer, Weitering & Vonk, 1976). At maximal DBSP excretion,pentobarbital treatment did not significantly change bile flow (Table I), but during the last part of the experiment bile flow was increased. Rutishauer and Stone (1975) compared the effect of pentobarbital and urethane anesthesia in rabbits and observed a higher bile flow in pentobarbital anesthetized animals. Cooper et al. (1976) did not find a significant increase in bile flow after pentobarbital administration in rats under restraining conditions. In the present study Tm, Cm, r_{23} and recovery of DBSP in bile were decreased after pentobarbital treatment, while primary hepatic clearance constant was not changed. This effect of pentobarbital on biliary excretion of DBSP may be caused by competition of pentobarbital and/or its metabolites with DBSP in the biliary excretion process. In rats pentobarbital and metabolites are excreted in urine (Ossenberg, Peignoux, Bourdiau & Benhamou, 1975), but also in bile (Klaassen,

1971). Bailey, Paul & Johnson (1975) reported an inhibitory effect of pentobarbital on hepatic transport of succinylsulfathiazole, when they compared the influence of urethane and pentobarbital. Cooke & Cooke (1977) observed an inhibitory effect of pentobarbital compared with urethane on hepatic transport of iopanoate glucuronide.

In our experiments during fasting many pharmacokinetic values were changed. Bile flow and Tm of DBSP were decreased, which confirms the results of Mahu et al. (1977), who described that 72 h of fasting diminished bile flow, bile salt output and Tm of DBSP. A retarded plasma disappearance of DBSP during fasting was also observed by Stein, Mishkin, Fleischner, Gatmaitan & Arias (1976), who correlated this phenomenon with a decreased amount of Y protein in the liver. During fasting pharmacokinetic parameters in the uptake and biliary excretion process were changed simultaneously, which hinders a clear analysis of changes of DBSP transport.

The hepatic transport of DBSP was studied in three different groups of rats: the permanent bile fistula rats, the acute bile fistula rats and the non bile fistula rats. In the permanent bile fistula rats no portal supply of bile salts to the liver occurs, due to the interrupted enterohepatic circulation. The possible influence of bile salts on primary hepatic uptake of drugs, is therefore ruled out. The data of the permanent bile fistula rats were compared with those obtained from the acute bile fistula rats to study the chronic effects of the recirculating bile salts. In acute experiments a part of the bile salts from the total bile salt pool disappeared due to the interruption of the enterohepatic circulation for about one hour. In the acute bile fistula rats during pentobarbital anesthesia compared with the permanent bile fistula rats during pentobarbital anesthesia many parameters were changed. The V_c was smaller, which may be caused by a different surgical procedure, but the primary hepatic clearance constant was not significantly changed. This indicates that the hepatic uptake of DBSP is not influenced by bile salts present in portal blood. The higher r_{21} in the acute bile fistula rats is difficult to explain. The biliary excretion process was markedly

different: bile flow, Tm and C_m of DBSP excretion were higher in acute bile fistula rats. These differences cannot be caused by the recirculating bile salts alone, because total output of bile salts was not significantly different in both groups of animals (Vonk et al., 1978[c]).

To study both the chronic and the acute effects of bile salts on pharmacokinetics of DBSP, the data of the non bile fistula rats at noon were compared with those from the permanent bile fistula rats at noon and the data from the non bile fistula rats during pentobarbital anesthesia with those from permanent bile fistula rats during pentobarbital anesthesia. From both comparisons, it can be concluded that the primary hepatic clearance constant is not different, but the biliary excretion (r_{23}) is distinctly higher in rats with an intact enterohepatic circulation. One of the reasons for this increase in r_{23} may be a high bile flow due to the recirculating bile salts.

This observation seems to be in contrast with the observation of Eriksson, Hellström & Ryrfeldt (1975), who studied the biliary excretion of ^{3}H-terbutaline, in unanesthetized rats with an intact enterohepatic circulation and observed that the biliary excretion in those animals was not much different from that in anesthetized animals. However, biliary excretion of this organic cation is not expected to be changed by an anionic anesthetic agent like pentobarbital and/or metabolites, while biliary excretion of most organic cations is not dependent of bile salt output (Vonk, Scholtens, Keulemans & Meijer, 1978[b]).

The bile salt concentration in portal blood will be much lower in the permanent bile fistula rats than in both other groups of rats. In spite of this,the primary hepatic uptake of DBSP was not significantly different, which is in contrast with the observation of Marinovic et al. (1977), who suggested that bile salts facilitate hepatic uptake of DBSP in the dog. The present data also do not indicate any inhibitory effect of bile salts on hepatic uptake of DBSP, as reported previously (Vonk et al., 1978[a]). This inhibitory effect depends on the bile salt concentration and was observed in isolated hepatocyte suspensions with concentrations higher than 40 µM. In the present studies no

portal bile salt concentrations were measured. Cronholm & Sjövall (1967) reported that the portal bile salt concentration in rats amounted to 60 μM. According to our previous studies this concentration would be expected to cause small inhibitory effects on hepatic uptake of DBSP. The discrepancy may be explained by animal differences, diet differences (Cronholm & Sjövall, 1967), or mixing of portal blood with arterial blood, resulting in lower sinusoidal bile salt concentrations.

This study indicates that the biliary excretion of DBSP varies with the time of the day and is influenced by pentobarbital anesthesia, food deprivation and interruption of the enterohepatic circulation of bile salts.

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SAMENVATTING

Het effekt van galzouten op het transport van farmaka via de lever werd in ratten onderzocht. De twee belangrijkste interaktie-niveaus bleken te zijn:

a. het galuitscheidings-proces

b. het leveropname-proces

Het effekt van galzouten op de *galuitscheiding* van farmaka was niet éénduidig: de galuitscheiding van bepaalde farmaka werd gestimuleerd, terwijl de galuitscheiding van andere farmaka niet werd aangetast. Binnen de laatste kategorie zijn er farmaka, waarvan de leveropname de snelheidsbepalende stap voor het transport van plasma naar gal bleek te zijn. Het mechanisme van de verhoging van de galuitscheiding van farmaka is afhankelijk van het onderzochte farmakon: de galuitscheiding van dibromosulphthalein is afhankelijk van de galstroom op zich, terwijl de galuitscheiding van indocyanine groen en rose bengal niet direct gerelateerd is aan de galstroom.

De remming van *leveropname* van farmaka door galzouten, aangetoond in geïsoleerde hepatocyten, geïsoleerde doorstroomde ratte levers en in intacte ratten, werd waargenomen bij relatief hoge galzout spiegels in het plasma (>100 µM). De leveropname van zowel positief als negatief geladen farmaka was geremd, hetgeen kompetitie fenomenen minder waarschijnlijk maakt. Deze remming van leveropname van sommige farmaka kan farmakokinetische en farmakotherapeutische konsekwenties hebben, vooral in die pathologische situaties, waarin hoge serum-galzout-koncentraties voorkomen. Farmaka, die gewoonlijk in grote mate door de lever worden opgenomen, dienen daarom in genoemde pathologische situaties zorgvuldig gedoseerd te worden, ook indien deze farmaka niet in de gal uitgescheiden worden.

Galzouten, die in physiologische omstandigheden in ratten afkomstig zijn van de enterohepatische kringloop, stimuleren de galuitscheiding van sommige farmaka, terwijl de leveropname van die farmaka niet is veranderd.

CURRICULUM VITAE

Roel J. Vonk, geboren op 23 maart 1947 te Baflo (Gr.), bezocht van 1959-1960 de Chr. Mulo-school te Uithuizermeeden en van 1960-1965 de 1e Chr. H.B.S. te Groningen. Hij studeerde van 1965-1972 scheikunde aan de Rijksuniversiteit van Groningen met als hoofdvak biochemie en als bijvak technische chemie. Sinds oktober 1972 is hij werkzaam op het Farmacologisch Laboratorium te Groningen en betrokken bij onderzoek en onderwijs op het gebied van de farmacokinetiek.

Hij is gehuwd en heeft een dochter.

Uit onderzoekingen, waaraan is meegewerkt, zijn de volgende publikaties voortgekomen:

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